THE NITROGEN METABOLISM OF THE LEAVES OF *ATRIPLEX NUMMULARIUM*

by

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INTRODUCTION.

In a previous paper (Wood, 1932) it has been shown that in *Atriplex* species the carbohydrate metabolism is in part diverted to form mucilaginous pentosans with a high hydration capacity. These confer drought-resistant properties on the plants to a certain extent.

Mothes (1930) showed that the water-retaining capacity of protein-poor leaves of *Phaseolus multiflorus* was less than in protein-rich leaves of the same species. He concluded that with increasing water-loss proteolysis occurred more rapidly, and that drought-resistant species showed only small proteolytic activity. Mothes found only a slight difference in the soluble nitrogen fractions between turgescent and wilted leaves, but thought that this difference was significant.

The work to be described here was designed in the first instance to trace the changes in the nitrogen metabolism of species of *Atriplex*. These plants, commonly known as saltbushes, are found in the driest habitats of arid Australia, especially between the 8 and 5 inch isohyets (Osborne, Wood, and Paltridge, 1932). *Atriplex nummularium* Lindl., commonly known as the "Old Man Saltbush", was chosen as experimental material on account of the large size of its leaves, which permit of half-leaves being used as controls. It is not one of the most drought-resistant species of this genus, but is found usually near permanent water-tables in the dry areas.

In view of certain apparently contradictory statements in the literature dealing with nitrogen metabolism, it appeared desirable to investigate the equilibria existing in the leaf between the various nitrogen-containing compounds.

Mothes (1925) gives a historical survey of the literature dealing with the various nitrogen fractions in leaves. The modern work of Schulze (1906), Butkewitsch (1909), Prianischnikow (1924), Smirnow (1923), and Mothes (1925) has shown that hydrolysis of proteins occurs, resulting in the formation

of amino-acids. Then follows an oxidative change of these degradation products resulting in the formation of ammonia. In carbohydrate-poor plants the ammonia accumulates as ammonium salts of organic acids such as oxalic, malic, and succinic acids, and may accumulate in such quantity as to cause ammonia poisoning in the leaves. In carbohydrate-rich leaves, on the other hand, asparagine, the monamide of aspartic acid, accumulates.

Butkewitsch (1900), following the suggestion of Cl. Bernard (1878) that during narcosis all synthetic processes come to a standstill, found, in anaesthetized leaves which normally accumulate asparagine, that not asparagine but large quantities of ammonia were formed. Mothes (1928) showed conclusively that asparagine was formed as a secondary process from ammonia.

Mothes (1928) found that amides increase in leaves in the dark. Engel (1929), on the other hand, found no increase in the amide content of starved leaves, but found that the amino-acids increased. In the roots, on the contrary, the amides increased considerably.

Ruhland and Wetzel (1926, 1927) divided plants into "acid plants" and "amide plants". In the former organic acids and ammonia accumulate from the deaminization of amino-acids; *Rheum* and *Begonia* are typical examples. In "amide plants" asparagine is present in quantity as a reserve stuff. *Asparagus* is a characteristic amide plant.

According to Pfeiffer (1925), *Rheum* and *Begonia* have an acid cell sap of pH 3–4. *Asparagus*, on the other hand (Rohde, 1917), has an alkaline sap of pH 7·2.

These facts suggest an enzymic control of the protein degradation products, in which the relative amounts of amino-acid, ammonia, and amide are determined by the hydrogen ion concentration, and this aspect has received consideration here.

METHODS.

All analyses were carried out with mature leaves obtained from the same bush growing on the banks of the River Torrens near Adelaide.

For the analyses of the nitrogen-containing compounds from 6 to 10 gm. of the fresh leaves were finely ground with pure washed sand and a little water in a porcelain mortar. For the precipitation of proteins, etc., the usual technique followed in blood analyses was adopted. To the ground leaf paste 5 c.c. of a 10 p.c. sodium tungstate solution and 5 c.c. of 2/3 N sulphuric acid were added drop by drop. Sufficient water was then added to bring the total volume of solution, including the water present in the leaves, up to 50 c.c. The water content of each sample of leaves was determined, and any necessary readjustment allowed for. Tungstic acid proved the most effective precipitant and permitted rapid filtration. Uranium acetate gave clear filtrates only after many hours' standing. Tannic acid, as used by Mothes (1925) gave rapid filtration and accurate results for ammonia and amides, but introduced large errors in the estimation of amino acid N by the Van Slyke (1912) method, since the tannin oxidizes on standing, and gives off considerable quantities of oxygen on diazotization, and this can be removed from the apparatus only with difficulty.

After the addition of water and the tungstic acid solutions the mixture was filtered. The time from the cessation of grinding to filtration was 15 minutes. Aliquots of the filtrate were then used for the determination of ammonia-N, amide-N, and amino-N.

Ammonia was determined on 15 c.c. of the clear filtrate by distillation with lime-water in a Claisen flask under reduced pressure. An air-leak was provided by a fine capillary tube through one arm. The mixture boiled between 26° and 30°C. An air condenser only was used, at the end of which was a Folin absorbing bulb. The NH_3 was absorbed in N/10 H_2SO_4 and estimated colorimetrically by Nesslerization.

The amide–N was estimated in the same solution by acidifying with H_2SO_4 to make a 2 p.c. solution and hydrolizing for 2 hours. This operation was performed in the Claisen flask, and the NH_3 formed was determined as before.

At first the amino-N was determined in the remaining solution, acidified with acetic acid. Trials showed, however, that there was no difference between the amino–N found in this solution and that of the original filtrate. It is known that amide-N is not affected by the Van Slyke treatment, and the NH_3 content is so small that no error is introduced during the 5 minutes deaminization in the bulb of the apparatus.

Protein-N was determined at first by the Kjeldahl procedure on the residues precipitated by tungstic acid. This includes also the N from chlorophyll, which is negligible. Later the total N was determined by Kjeldahl on the dried and ground leaf powder used for the water estimation. The protein-N was then obtained by deducting the NH_3-N, amide-N, and amino-acid N from this figure. This saved the somewhat difficult washing of the precipitate and diminished the time of digestion considerably by allowing the use of smaller quantities. The figure for protein-N by this method is not as accurate as by the first, but protein estimations were not required for any critical experimental purposes.

The pH values of the leaves were determined *in situ* by the use of a platinum microhydroquinone electrode. A steady E.M.F. was obtained after about 3 minutes. The technique consisted in lightly scraping the upper surface of the leaf with a glass scraper; hydroquinone was added, and a bright platinum strip placed over the scraped area. The part of the leaf was then imprisoned between two glass microscope slides and clamped to prevent undue loss of CO_2. Contact was made through the leaf by placing an agar–KCl tube in contact with another lightly scraped surface.[1]

All results are expressed in percentage of the fresh weight of the leaves unless otherwise indicated, since the work of Chibnall (1923) and Mothes (1925) was carried out on this basis, which shows less variation, especially in starvation experiments, than a dry weight basis. In cases where wilting occurred the N fractions are expressed on a crude fibre basis (Rahn, 1923).

THE NORMAL COURSE OF NITROGEN METABOLISM IN THE LEAVES.

Table 1 shows the amounts of the different N-fractions in mature leaves about one year old, picked directly from the bush at 9.00 a.m. Twice the amide-N gives a measure of the asparagine and glutamine, and the amino-acid N is obtained by deducting the amide-N from the total amino-N.

The ammonia-N remains practically constant throughout, the amide and amino-N fractions decrease from the middle of September. This time marks the end of the cold season and also the onset of growth, new leaves appearing about

[1] I am greatly indebted to Mr. J. W. H. Lugg who kindly made the pH determinations.

the middle of September. Fluctuations in the amide-N content are to be expected in attached leaves since it is transported. It will be shown in a later section that the decrease in amino-acid-N is due to a shift in the equilibrium between protein and amino-acid by temperature.

TABLE 1.

Nitrogen Fractions in Normal Mature Leaves.

Date. Aug.	Temp. °C	H_2O % F.W.	Dry Matter % F.W.	NH_3–N % F.W.	2-amide N % F.W.	amino-acid-N % F.W.	Protein-N % F.W.
16	14	85·40	14·60	·0010	·012	·016	·256
21	14	84·91	15·09	·0015	·014	·015	·255
30	14	84·71	15·29	·0015	·006	·015	·320
Sept.							
6	13	87·00	13·00	·0012	·010	·014	·385
11	14	84·20	15·80	·0017	·007	·015	·387
Oct.							
13	17	87·40	12·60	·0018	·002	·010	·380
18	20	88·00	12·00	·0020	·002	·008	·350
Aug.							
24	14	81·38*	18·62	·0010	·010	·037	·233

*Young immature leaves.

The protein content at first increases, supporting Mothes' conclusion (1930) that the old leaves of evergreen species behave as young leaves.

In young attached leaves the normal ammonia and amide content is the same as in mature leaves, but the amino-N content is higher.

In normal leaves the mean of many determinations gives a value of pH 6·48, the extreme ranges observed being between 6·41 and 6·71.

During starvation in darkened, mature leaves of *Atriplex* attached to the shoot, the protein-N shows a progressive decrease. The amide-N and amino-N remain at approximately the same level as in normal leaves. The ammonia-N, however, shows a decided increase. Table 2 shows such a series.

TABLE 2.

Nitrogen Fractions in Darkened Leaves Attached to Shoot.

Date. Aug.	Temp.	Hrs. in dark.	H_2O	Dry matter.	NH_3–N	2-amide-N	amino-acid-N	Protein-N
21	14°	control	84·91	15·09	·0015	·014	·015	·255
25	14°	96	82·15	17·85	·0026	·014	·015	·204
28	14°	168	82·31	17·69	·0023	·010	·015	·165

In detached leaves standing in the dark, with their petioles in water, the increase in the ammonia N is more marked, as Table 3 shows.

TABLE 3.

Nitrogen Fractions in Darkened Mature Leaves, cut from bush, and with Petioles Standing in Water.

Date Sept.	Temp.	H_2O	Dry Matter.	NH_3–N	2-amide-N	animo-acid-N	Protein-N
6	Control 13°	87·00	13·00	·0012	·010	·014	·385
8	13°*	87·00	13·00	·0038	·013	·013	·390
11	Control 14°	84·20	15·80	·0017	·007	·015	·387
13	30°*	84·20	15·80	·0093	·016	·011	·380
Oct.							
13	Control 16°	87·40	12·60	·0018	·004	·010	·380
23	18°*	87·00	13·00	·0150	·015	·003	·358
16	Control 20°	87·10	12·90	·0020	·004	·008	·350
20	18°*	87·00	13·00	·0075	·010	·005	·330

*Leaves kept in constant temperature incubator over period.

During the starvation period of the cut leaves the pH remains approximately constant, measurements giving a range between 6·48 and 6·44. In addition to the increase in ammonia-N the amide-N also increases, but not to the same extent as the NH_3-N. This is doubtless due to the low carbohydrate content of the leaves of *Atriplex*. Mothes (1925) has shown clearly that increased amide production occurs in carbohydrate-rich plants. Unlike drought-resistant plants of the sclerophyll type, *Atriplex* spp. show a restricted carbohydrate content, the bulk of the dry matter consisting of protein and ash. Table 4 shows an analysis of the leaves of *Atriplex nummularium*, and also for comparison *Hakea ulicina*, a typical plant with sclerophyll leaves. All figures are expressed as percentage of fresh weight.

TABLE 4.

	Water	Dry Matter	Monosaccharides	Disaccharides	Starch	Pentosans	Total respirable carbohydrates	NH_3–N	2-amide N	amino acid N	Protein N	Alkali Sol Resins	Cellulose	Lignin	Cutin	Tannin	Ash
A.	85·40	14·6	·088	·176	·062	·375	·701	·001	·010	·015	·40	2·00	·473	·032	·068	—	3·83
B.	47·60	52·4	1·200	·991	·610	4·76	7·56	·004	·007	·001	·39	7·69	10·2	3·52	7·36	2·8	2·09

A=*A. nummularium*
B=*H. ulicina*

CHANGES IN NITROGEN METABOLISM DURING WILTING.

It has been pointed out previously that the leaves of *Atriplex* spp. resist permanent wilting to a marked degree. During prolonged wilting the protein con-

tent of the leaves decreases. Table 5 shows the changes in leaves attached to a shoot which had been allowed to wilt in the dark for 14 days. The ends of the shoots were almost covered with paraffin wax and the shoots placed in water. This procedure permitted wilting to proceed fairly slowly. During this period in darkness new leaves were produced from axillary buds, obviously at the expense of the wilted leaves, as may be seen from Table 5.

In Table 5, *A* gives the analysis of leaves immediately after the shoots had been picked; *B*, that of leaves which had been allowed to wilt over the period of 14 days; *C*, that of the young leaves produced during the period. Owing to the gross changes in water content the protein-N is expressed as percentage of the crude fibre.

TABLE 5.

Changes in Protein-Nitrogen during Wilting.

	Date.		Temp.	Water.	Dry Matter.	Soluble N	Protein N
A.	Aug.	30	14°	85·92	14·08	·040	73·7
B.	Sept.	12	14°	74·78	25·22	·050	54·5
C.	„	12	14°	79·00	21·00	·072	145·4

During the early stages of wilting there is little change in the nitrogen content of the leaves compared with unwilted leaves. At this stage the leaves recover turgidity when placed in water. With greater water-loss, however, the leaves become permanently wilted and the production of ammonia and amide-N increases considerably. These changes are shown in Table 6. The leaves were allowed to wilt for 24 hours. The amounts of various N-fractions are expressed on the original fresh weight of the leaf. A is a control leaf picked directly from the bush, B represents wilted leaves, and C permanently wilted leaves.

TABLE 6.

Nitrogen-Fractions in Cut, Wilted Leaves.

	Date. Oct.	Temp.	Water.	Dry Matter.	NH_3–N	2-amide N	Amino acid N	pH Surface.	pH Deep.
A.	18	20°	87·0	13·0	·0021	·004	·008	6·41	6·68
B.	19	20°	80·0	20·0	·0027	·008	·009	6·17	6·40
C.	19	20°	69·0	31·0	·0044	·018	·007	7·86	7·10

During wilting the hydrogen ion concentration in the leaves varied. In leaves which were not permanently wilted the sap was more acid than in the normal, unwilted leaves. In permanently wilted leaves the sap was definitely more alkaline. In Table 6 pH values are given for both light surface scratchings and for deep cuts into the central assimilating tissue.

THE EFFECT OF pH ON THE AMOUNT OF THE VARIOUS NITROGEN FRACTIONS IN LEAVES.

The changes in hydrogen ion concentration taken in conjunction with the N-changes during wilting also suggest a connection between pH and enzymic activity in the leaf.

Preliminary experiments showed that there was no change in the actual amount of proteolytic enzymes during wilting. Shoots of *Atriplex* were left in the laboratory in water, but with the cut ends partially stopped with wax. After 4 days the lower leaves were wilted, but the upper ones were still turgid; 10 gm. of each were ground and allowed to autolyse in sterile flasks for 48 hours at 30°C. The total volume was brought up to 50 c.c. in each case and the pH adjusted to pH 6·0 by McIlvaine's (1921) phosphate-citrate buffers. Table 7 shows that there was no increase in amino-N over this period.

TABLE 7.

Proteolytic Activity of Turgid and Wilted Leaves.

	Water. % F.W.	Dry Matter. % F.W.	Protein N. % Raw Fibre.	Amino-N in 10 c.c. (mg.). Start.	Finish.	Difference.
Control	87·00	13·00	72·5			
Turgid	85·13	14·87	73·8	·2085	·4734	·265
Wilted	80·59	19·41	53·3	·3165	·5934	·276

There remained therefore the possibility of varying activity of the enzymes at different pH values.

To demonstrate the effect of hydrogen ion concentration, 6 gm. of the fresh picked leaves were finely ground with pure sand and 20 c.c. of McIlvaine's (1921) phosphate-citrate buffers and sufficient water added to bring the volume (including the water in the leaf tissues) to 50 c.c. This was poured into sterilized flasks; 0·5 c.c. of a 50 p.c. chloroform-toluol mixture was added and the flasks incubated for various periods at 30°C. After incubation 20 c.c. of the well-shaken mixture were removed, and 5 c.c. each of sodium tungstate solution and 2/3 N H_2SO_4 added to each lot.

After filtration 10 or 15 c.c. of the filtrate were hydrolized for 2 hours with 2 p.c. H_2SO_4 and the combined NH_3 and amide-N determined colorimetrically. The remaining filtrate was utilized for amino-N determinations by Van Slyke.

A half-leaf method was used throughout, one half of the leaves being used for the most acid buffer and the other half for the most alkaline. Similarly for all intermediate values the two halves of each batch were utilized for extreme values.

Preliminary experiments showed that there was practically no further amide production during autolysis, ammonia accumulating as such.

Table 8 shows the amounts of NH_3-N and amino-N produced per 20 c.c. solution (equal to 2·4 gm. fresh weight of the leaves) at different pH values after 4 days' autolysis. These results are also shown graphically in fig. 1.

TABLE 8.

Increase in Nitrogen-Fraction during Autolysis at 30°C.

pH	NH_3–N	Amino N	Total soluble N.
9·0	1·231	—	1·231
8·2	1·143	·032	1·175
7·5	·947	·153	1·100
5·6	·621	·451	1·072
4·1	·250	·974	1·234
3·5	·200	·869	1·079
2·6	·150	·700	·850

These figures show clearly that the amino-N increases with increased acidity up to a point, whilst ammonia production is at an optimum at pH 8 to 9.

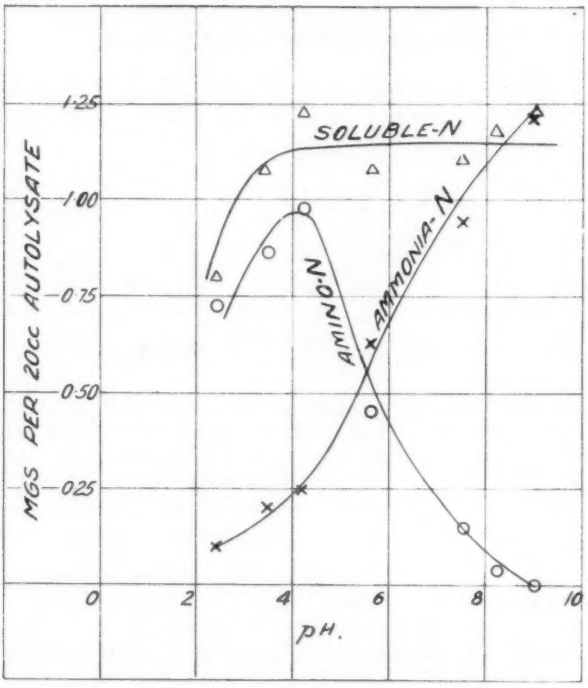

Figure 1. Influence of reaction on the activity of protease and deaminase in *Atriplex* leaves.

This points clearly to the presence of a deaminase in the leaf, destroying amino-acids. At low pH values very little ammonia production occurs. It follows that the amino-acid content of a leaf at any stage is not a measure of the

amount of proteolysis, but the sum of the amino, ammonia, and 2-amide N does give a measure of this. This value, the "soluble N" of the leaves, remains practically constant at all pH values between about 3·5 and 9·0, as can be seen from Table 8. Below pH 3·5 and above pH 9·0 the amount of soluble nitrogen produced during autolysis is depressed.

The extended range over which proteolysis is at an optimum suggests that a mixture of proteases is present in the leaf of both peptic and tryptic types. The autolysis of broad bean leaves (Table 11) suggests that a similar mixture of enzymes occurs here also, though it is not necessarily universally present in leaves.

At values above pH 6·5 (the normal value in *Atriplex* leaves) the amino-N may drop below the level present in the leaf, owing to the activity of the deaminase at these hydrogen ion concentrations. This decrease in the amount of amino-N over the critical pH range is illustrated in Table 9, which shows analyses of 6 gm. of leaves after 2 days' autolysis. This was a preliminary experiment, in which the NH_3-N was determined.

TABLE 9.

pH	Amino-N (mg. per 20 c.c.)
Originally present	1·0050
8·3	·0894
7·9	·4338
6·9	·6366
6·6	1·0140

At about pH 5·5 the amounts of amino-N and NH_3-N are equal and will increase equally. Above or below this value either amino-N or (NH_3+amide)N will be in excess in the leaf. The reason for the division into "acid" and "amide" plants made by Ruhland and Wetzel (1925) is now clear. "Acid plants" such as *Begonia*, *Rheum*, and *Oxalis*, have pH values between 3 and 4·5 (Rhode, 1917; Pfeiffer, 1925), and "amide" plants, such as *Asparagus officinalis*, has pH of 7·2, *Vicia faba* a pH of 6·9 (Pfeiffer). The amount of deamination in different leaves will depend, not only on the pH value, but also, obviously, on the amount of deaminase present.

It is interesting to note that Small (1929), in his general survey of the pH of the tissues of Angiosperms, differentiated on a quite arbitrary basis, "acid" families as having a pH below 5·3, and "alkaline" families with a pH above 5·5.

Confirmatory analyses showing the changes in the N-fractions with varying hydrogen ion concentrations are shown in Table 10. The series A is an autolysis carried out as before at 30°C. for 24 hours, and series B a similar autolysis at 30°C. for 13 days.

TABLE 10.

Changes in Nitrogen-Fractions during Autolysis at 30° C. for 24 hours and 13 days.

	pH	NH_3–N	Amino-N	Soluble N.
Series A.		per 20 c.c.		
	2·4	·1052	·617	·722
	4·0	·1152	·634	·749
	5·0	·1251	·609	·734
	8·0	·2143	·475	·690
Series B.				
	2·4	·1000	·186	·286
	5·0	1·4630	1·68	3·14
	8·0	2·1816	1·15	3·33

A similar relation between amino-N and NH_3-N exists in *Vicia faba*. In Table 11 the changes in these N-fractions are shown. Four gm. of leaves were autolysed for 3 days under the usual conditions.

TABLE 11.

Changes in Nitrogen-Fractions during Autolysis in Vicia faba.

pH	NH_3–N	Animo-N.
3·3	·330	·62
6·5	·450	·34
8·0	·817	·10*

**Note.* In *Vicia faba* oxidases are active and a black-coloured pigment is produced at pH>7. This compound gives off O_2 in the Van Slyke apparatus which is measured as N unless absorbed with alkaline pyrogallol.

By altering the pH of the sap of entire leaves of *Atriplex*, changes in the predicted directions were observed. Large changes in pH were not obtained, owing to the efficient buffering system present in the leaf. In the experiment shown in Table 12, cut leaves were placed with their petioles in acidulated water at pH 2·0 in one case (A), and in the other (B) in water made alkaline to pH 10 with Na_2CO_3. C was a control leaf in distilled water. The leaves were placed in the dark for 4 days and then analysed. The pH values of the leaves were 5·98, 7·00, and 6·48 respectively.

TABLE 12.

Changes in Nitrogen-Fractions of Cut Leaves at different pH values.

	pH	(NH_3+2-amide) N	Amino acid N	Soluble N.
A.	5·98	·018	·010	·028
B.	7·00	·022	·004	·026
C.	6·48	·018	·005	·023

The increase in ammonia-N and amide-N in cut leaves of *Atriplex* is easily understood in the light of the above experiments, since the pH of the sap is about 6·5. The apparently contradictory results obtained by Engel (1929) with the roots and leaves of *Zea mais* are similarly explicable. In the leaves, which are relatively acid, amino-acids accumulate, but in roots, which are invariably more alkaline, amides predominate.

A survey of the results of Mothes (1925, 1930), Smirnow (1928), Grüntuch (1929), and Rahn (1932) shows clearly that when there is an increase in (NH_3+amide)N there is a corresponding decrease in amino-N, and *vice versa*. Most of the fluctuations shown in the N-fractions in progress curves can probably be traced to fluctuations in pH values.

The influence of hydrogen ion concentration has a considerable bearing on the problem of senescence in plants. Mothes (1925) and Smirnow (1928) have shown that protein disappears rapidly from senescent leaves. Fluctuations in hydrogen ion concentration will not affect the rate of proteolysis judging from the leaves examined here.

An excess of ammonia or amide above the amount in equilibrium with amino-acids represents an excess which is capable of being transported from the leaf; this occurs at high pH values. Similarly at low pH values an excess of amino-acids above the amount in equilibrium with protein may occur, and this excess may also be transported from the leaf.

The amounts of ammonia, amides, and amino-acids present in the leaf at any time represent a balance between their rate of formation and destruction on the one hand and the rate of transportation on the other. Minimum loss would occur in a leaf at about pH 5·5, where the balance between transportation and protein degradation resulted in the presence of approximately equal amounts of amino-acid and (ammonia + amide). A change in pH value on either the acid or the alkaline side would result in increased production of degradation products capable of being transported, and their removal causes further protein degradation. Unless this is made good by protein synthesis the leaf becomes senescent by loss of its protoplasmic proteins.

The changes in hydrogen ion concentration with maturity indicate that either an increase or a decrease in pH may occur. Haas (1920) records a decrease in pH from 5·8 to 5·3 in maize seedlings with increasing age. Hempel (1917) records a rise from pH 5·78 to 6·03 in the lupin. Haas (1920) and Gustafson (1924) show a gradient from more acid young leaves to less acid old leaves in several species. Hurd (1924) found in wheat that in early months of growth the pH values lie between 6·3 and 5·9, but in the later stages falls to pH 5·6. Smirnow (1928) in tobacco records a fall from pH 6·0 in the seedling stage to pH 5·3 in the ripe leaf; in the sunflower, on the other hand, there is an increase from pH 7·4 in the seedling to pH 7·9 at maturity. Smirnow also gives analyses for amino-N, amide-N, and NH_3-N, and examination of this data shows

an increase of amino-N with increased acidity, and increased (amide+NH_3)N with decreased acidity.

In *Atriplex nummularium* there is a slight increase in pH with increasing age of the leaf. Young expanding leaves have a pH of 6·25, expanding semi-mature leaves 6·31, mature leaves 6·48, and old yellowing leaves 6·60.

The changes in pH with increasing age are probably correlated with changes in the components of the buffer systems in the leaves.

The changes in hydrogen ion concentration during wilting and the resulting effects on nitrogen metabolism in the saltbush are of considerable interest. As shown in Table 6, the pH of normal turgid leaves is 6·48, wilted leaves are more acid with pH 6·17, and permanently wilted leaves are definitely alkaline with pH 7·86. The small increase in acidity in the wilted leaves results in a slowing up of the protein loss, since less amide+ammonia is produced. The high pH in permanently wilted leaves permits rapid deaminization of amino-acids, with consequent further hydrolysis of protein to maintain equilibrium. The increase in acidity, or even the maintenance of the normal pH value, is therefore a shift towards protein conservation, and therefore of water conservation. Mothes (1930) has shown protein-rich leaves have a higher hydration capacity than protein-poor leaves.

The increase in acidity at the first wilting is probably due to increased CO_2 production by respiration, since Iljin (1923) and Collorio (1928) have shown that the respiration increases with wilting. The increase in acidity would favour also the loss of starch by hydrolysis which has been observed during wilting by Iljin (1923), Ahrns (1924), and Lundegardh (1914).

The increased pH values during permanent wilting in *Atriplex* is doubtless caused by the increased concentration of the various substances acting as buffers in the leaves. Buffer indices have not been determined, but the presence of efficient buffers, probably of phosphates, can be gauged from the changes in pH of the phosphate-citrate buffers by the addition of the ground leaf material.

In *Atriplex nummularium* oxalic acid and malic acid are both present as calcium salts, the former being present in large quantity in the hypodermal cells of the leaf. Phosphates are present, the average amount being 0·14 p.c. P_2O_5 of the fresh weight. Proteins form about 2·5 p.c. of the fresh weight of the leaf. Oxalates buffer between pH 3·0 and pH 5·3; malates between pH 3·7 and pH 6·0. In *Atriplex* therefore both these acids will exist as inactive Ca salts. Inorganic phosphates buffer between pH values of 5·2 and 8·0. A bicarbonate-CO_2 system also is possibly present in the leaf, with a maximum buffer capacity at about pH 7·0. Beyond pH 6·0, however, in the direction of greater acidity any carbonate will exist as free acid. In *Atriplex*, phosphates, together with protein, probably play the major part as buffers, and a shift of pH in the direction of greater acidity is conceivable, due to increased respiration on wilting. Further work is necessary to trace the cause of the increased alkalinity in permanent wilt-

ing, which is probably due to changes in concentration of the components of the buffer system.

Maximow (1929), Zalenski (1923), and Alexandrow (1922) have shown that the upper leaves of a shoot transpire more rapidly than the lower leaves. Yapp and Mason (1932) record a "rapid loss of water from older leaves, and a comparatively insignificant loss (in some cases an apparent gain in the terminal bud), which suggests a transference of water from the older to the younger leaves". In *Atriplex* there is an obvious visible loss of water from the lower leaves, the upper ones remaining turgid. The mechanism of wilting in the lower, older leaves is therefore clear, and any process tending to restrict the loss of protein from these leaves until turgidity is restored must be regarded as a drought-resistant feature. The increased acidity during wilting is such a feature, but one which is probably by no means restricted to plants such as *Atriplex*.

A feature of more importance in protein conservation is probably the concentration of protease in the different leaves. The amount of soluble nitrogen does not bear any simple relation to the amount of protein-N in leaves, but depends also on the enzyme concentration. Mothes (1930) has indicated that relatively drought-resistant plants like *Rhododendron*, *Hedera*, and *Pinus niger*, show only small proteolytic activity. This is also the case with *Atriplex nummularium*. In an experiment with cut leaves of this plant, starved for 7 days, the soluble nitrogen increased only from 4·3 p.c. to 6·3 p.c. of the total N. In *Vicia faba*, under the same conditions, the soluble nitrogen rose from 9·0 p.c. to 33·0 p.c. of the total nitrogen.

THE EQUILIBRIUM BETWEEN PROTEINS AND AMINO-ACIDS.

The position of equilibrium between proteins and amino-acids in leaves is difficult to determine, owing to the transportation of the amino-acids on the one hand and their destruction by deaminases on the other. This equilibrium will not be affected by changes in hydrogen ion concentration, although the amount of synthesis will be determined by the pH. In protein syntheses *in vitro*, the optimum pH for synthesis is in general higher than that at which optimum hydrolysis occurs (Wasteneys and Borsook, 1930). In *Atriplex* hydrolysis occurs, as shown, over an extended range, but the optimum pH for synthesis is not known.

The position of equilibrium will, however, be shifted by temperature. From thermodynamic considerations (Moore, 1921) one would expect that a rise in temperature would increase the tendency towards synthesis, and consequently the amino-acid concentration would be lowered.

The constancy of the amino-acid content of *Atriplex* leaves at constant temperature is shown clearly in Tables 1 and 2. This concentration represents a balance between transportation and formation and deaminization of amino-acids. Its constancy in cut leaves as well as in attached leaves suggests strongly that it

represents also an equilibrium concentration with the reserve proteins of the protoplasm.

This is confirmed in part by the changes which occur in leaves kept for varying periods at different temperatures. Table 13 shows the changes in the N-fractions in cut leaves kept in the dark at 30°C. At this temperature there is a decrease in the amino-N such as would be expected on thermodynamic grounds.

TABLE 13.

Effect of Temperature on Nitrogen-Fractions in Cut Leaves.

Date. Sept.	Temp.	Water.	NH_3–N	2-amide N	Amino acid-N	Protein N.
6	13°	87·00	·0012	·010	·014	·385
8	13°	87·00	·0038	·012	·012	·397
Aug.						
30	14°	84·71	·0015	·006	·015	·320
31	30°	85·10	·0036	·012	·009	·311
Sept.						
11	14°	84·20	·0017	·007	·015	·387
13	30°	84·20	·0093	·016	·011	·380

The possibility exists that this change in amino-acid concentration might be due to increased activation of deaminases compared with proteases, or to increased transport facility with rise of temperature. Table 14 shows the changes in NH_3-N and amino-N in ground leaves allowed to autolyse at pH 8·1 at the temperatures indicated.

TABLE 14.

Effect of Temperature on Nitrogen-Fractions during Autolysis, expressed as mg. N per 20 c.c. solution (=2·4 gm. fresh weight).

Temperature °C	NH_3–N	Amino-N	Soluble N.
10	·1200	n.d.	
18	·1578	·1674	·3252
30	·1998	·2773	·4771
40	·2400	·336	·4736
56	·1665	0	·1665

If the NH_3-N, amino-N, and soluble N are plotted against temperature it will be found that the curves have approximately the same form and slope, with an optimum at about 40°C. Amino-acid production and deaminization proceed therefore at approximately the same rate, and the fall in amino-N with increasing temperature, shown in Table 13, cannot be ascribed to increased activity of the deaminase. Assuming the rate of transportation to remain constant with change of temperature, the change in amino-acid concentration represents a shift in the position of equilibrium in the direction of synthesis.

SUMMARY.

Investigations on the nitrogen metabolism of *Atriplex nummularium*, a drought-resistant Australian species, has shown that changes in hydrogen ion concentration profoundly affect the amounts of ammonia, amides, and amino-acids present in the leaves. Proteases show optimum activity over an extended range between pH 3·5 and pH 9. Below pH 5·5 amino-acids accumulate in cut leaves, but above pH 5·5 deaminization increases, with increasing pH, reaching an optimum at pH 8–9. Under these conditions ammonia and amides accumulate in the leaves.

The normal pH of the leaves of *Atriplex* is 6·5. During wilting this is lowered to about pH 6·2, but on permanent wilting it increases to pH 7·8. The effects of these changes in conserving protein and consequently water during wilting are discussed.

The normal course of nitrogen metabolism in the leaves and during starvation has been investigated. The rôle of hydrogen ion concentration and its effect on nitrogen metabolism during senescence are also discussed.

The equilibrium between amino-acids and protein in the leaves was also investigated, and the change in the position of equilibrium with temperature has been shown to be that expected from thermodynamic considerations.

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THE CONCENTRATION AND DISTRIBUTION OF PHOSPHORUS IN THE BLOOD OF MERINO SHEEP ON PASTURE

by

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It has been suggested by Malan, Green, and DuToit (1928) that the concentration of inorganic phosphorus in the blood of cattle is a useful indication of the sufficiency or deficiency of phosphorus in the country on which they are grazed. DuToit, Malan, and Rossouw (1930), from their observations on sheep on a basal diet, low in phosphorus, to which various amounts of phosphorus were added in an easily assimilable form, concluded that the concentration of inorganic phosphorus in the blood was a reflection of the amount of phosphorus in the diet, and that it was remarkably constant as long as the feeding conditions were unchanged. The observations of Martin and Peirce (1933) have confirmed this.

If the inorganic phosphorus of the blood is to be used as a criterion of the adequacy of the phosphorus in pastures, it becomes necessary to formulate standards both of its variability among individuals on the same pasture and of the variability that is likely to be found on different pastures. Marston (1928) found 3·6–5·3 mg., mean 4·5 mg., phosphorus per 100 c.c. of blood in 25 four-tooth wethers from the Middle-North of South Australia. Norris and Chamberlin (1929) recorded 4·1–7·5, mean 5·6 mg., for an indiscriminate assortment of 32 sheep at the Melbourne abattoirs. Rossouw (1930) reported 4·5–6·8, mean 5·1 mg., for 10 sheep on "ordinary rations" at Onderstepoort, South Africa. Bekker and Rossouw (1930) gave the higher value of 6·5 mg. as being normal for sheep in South Africa, but this was presumably based on the results obtained by Malan (1930a) and DuToit, Malan, and Rossouw (1930) on sheep to which bone-meal or sodium phosphate had been given. Auchinachie and Fraser (1932) found values varying between 4·4 and 6·4 mg., mean 5·1 mg., in 26 wether hoggets on pasture in Scotland.

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The main object of the work reported in this paper has been to extend these very meagre data, but the opportunity has also been taken to make some observations on the total concentration of phosphorus in the blood and its distribution, about which little work appears to have been done. Malan (1928a), (1928b), (1930b), reports single observations on seven sheep. From 13·9 to 20·5 mg. of phosphorus per 100 c.c. of blood were present, of which from 2·7 to 5·1 mg. were inorganic, 3·2 to 4·6 mg. were organic acid-soluble, and 7·0 to 10·8 mg. were lipoidal. The lower values were obtained from ewes with lambs. Rossouw (1930), in the 10 sheep on "ordinary rations" at Onderstepoort, found from 19·0 to 24·8 mg., mean 21·9 mg., of which 4·5 to 6·8, mean 5·1 mg., were inorganic, 3·9 to 7·5, mean 5·8 mg., were organic acid-soluble, and 9·9 to 11·8 mg., mean 10·9 mg., were lipoidal.

METHODS.

Collection of Blood.

Blood was taken from the jugular vein with a Record syringe lubricated with paraffin oil; a few c.c. were run into a test tube, the walls of which had been coated with potassium oxalate, and 5 c.c. were measured into 20 c.c. of 10 p.c. trichloracetic acid, and the mixture shaken and filtered within fifteen minutes. Except in the observations made in the North-East of South Australia, the sheep were brought in off pasture immediately prior to the withdrawal of the blood sample. In the North-East the sheep were bled in the field.

Estimation of Phosphorus.

The phosphorus was first converted into the inorganic form and then estimated according to the method of Fiske and Subbarow (1925).

Inorganic Phosphorus. These estimations were performed on the day of collection. They were made on the filtrate directly.

Total Acid-soluble Phosphorus. The filtrate was dry-ashed with a few drops of 10 p.c. magnesium nitrate at low red heat, the ash taken to dryness in dilute HCl and then taken up in sufficient H_2SO_4 to make the concentration in the final dilution for colorimetric comparison 0·5N. The phosphorus was determined in this solution, a 2·5 p.c. solution of ammonium molybdate in water being used in place of a 2·5 p.c. solution of it in 5N acid.

Total Phosphorus. Oxalated blood was dry-ashed with a few drops of magnesium nitrate, and the ash treated similarly to the filtrate ash.

The differences between the concentrations of inorganic phosphorus and total acid-soluble phosphorus, and of total acid-soluble phosphorus and total phosphorus have been reported as the concentrations of organic acid-soluble phosphorus and of organic acid-insoluble phosphorus respectively. It would appear from the remarks of Malan (1930b) that the organic acid-insoluble phosphorus of the blood of sheep is almost entirely lipoidal.

All concentrations have been reported as mg. phosphorus per 100 c.c. of blood.

RESULTS.

Observations on 65 sheep at the Waite Agricultural Research Institute, Glen Osmond, South Australia, at various times throughout the year are presented in Tables 1, 2, and 3. They are summarized in Table 4.

The mild wet winter season, characteristic of the area in which the Waite Institute is situated, prevailed in 1932, so that from mid-August to the end of October there was an abundance of feed, much in excess of the requirements of the sheep. During November and December little rain fell and the evaporation rate was high. As a result the excess spring herbage was converted into a standing straw. Sporadic rain of little value fell throughout January and February, 1933. Rains in March, sufficient to start growth, were supported by good rains early in April, so that by the middle of April a certain amount of young green herbage was available to the sheep. The pasture continued to improve throughout May and June, and in July there was an abundance of feed.

The observations set forth in Tables 1 and 2 were made on sheep on natural pasture. Analyses of samples of this pasture over a period of three years, recorded by Davies, Scott, and Fraser (1932), show that the phosphorus content of the dry matter rises to a maximum of about 0·20 p.c. of phosphorus during August and September, after which it gradually falls, and in January only 0·11 p.c. of phosphorus is present, while in late summer the amount may be less than 0·06 p.c. With the first winter rains there is a sharp rise in the amount present. During summer the sheep, by selection, are probably ingesting a fodder richer in phosphorus than the samples analysed.

In Table 5 some observations made on sheep in the North-East and Mid-North districts of South Australia are summarized. The North-East is a low-rainfall area, considered suitable for carrying about 40 sheep to the square mile. The staple food is saltbush, *Atriplex vesicarium*, though in some areas spear grass, *Stipa nitida*, predominates. Observations were made on sheep on a poor "stand" of saltbush, on a good "stand" of saltbush, and on spear grass. No green saltbush was visible in the poor "stand", and only a little green herbage in the bottom of the creeks. The sheep in this area were "in good heart", but their carcasses contained little fat. Bone-chewing was reported to occur amongst sheep on the spear grass.

The observations in the Mid-North were made following a warm spell after early winter rains. There was an abundance of young green feed. The natural pasture in this area is of a similar type to that at the Waite Institute. The top-dressed pasture contained a large proportion of legumes, particularly lucerne. The sheep on which the observations were made were all in good condition.

TABLE 1.

Concentration and Distribution of Phosphorus in the Blood of Wethers on Pasture at The Waite Institute, Glen Osmond, South Australia.

Dropped in the North-East of South Australia in 1928, received at the Waite Institute in 1929 as weaners, and since then maintained on this pasture.

Sheep.	Total phosphorus.		Inorganic phosphorus.		Total acid-soluble phosphorus.		Organic acid-soluble phosphorus.		Organic acid-insoluble phosphorus.	
No.	3/10/32	4/1/33	3/10/32	4/1/33	3/10/32	4/1/33	3/10/32	4/1/33	3/10/32	4/1/33
8	17·1	18·4	3·7	4·0	7·7	8·2	4·0	4·2	9·4	10·2
9	19·6	18·7	5·4	5·4	10·2	9·1	4·7	3·7	9·4	9·6
22	20·0	20·5	4·6	4·4	9·9	10·2	5·3	5·8	10·1	10·3
34	16·9	18·9	3·8	4·1	7·8	7·6	4·1	3·5	9·1	11·3
49	18·0	17·1	5·0	3·4	8·7	6·9	3·8	3·5	9·2	10·1
51	18·9	17·0	3·2	2·8	8·1	7·4	4·8	4·6	10·8	9·6
57	18·5	19·8	4·2	3·8	8·9	9·2	4·6	5·4	9·6	10·6
60	19·5	19·4	4·7	2·9	10·5	8·9	5·9	6·0	8·9	10·5
73	19·5	20·5	3·7	4·5	9·0	10·0	5·3	5·4	10·5	10·5
116	—	18·0	—	3·4	—	7·7	—	4·4	—	10·3
133	—	18·9	4·0	4·8	9·1	10·4	5·1	5·6	—	8·5
150	19·2	19·0	5·2	3·6	10·5	9·6	5·3	6·0	8·6	9·4
151	16·8	17·9	3·8	3·4	7·8	7·1	4·0	3·7	8·9	10·7
167	17·7	18·7	4·7	4·5	9·2	8·0	4·5	3·5	8·5	10·7
181	18·4	18·3	3·3	3·2	7·8	8·6	4·6	5·4	10·6	9·7
182	—	19·0	5·2	4·5	10·5	9·1	5·3	4·6	—	9·8
183	19·5	19·3	4·7	4·7	9·9	9·3	5·2	4·6	9·6	9·9
190	17·3	18·9	4·2	3·9	8·1	8·2	3·9	4·3	9·2	10·7
199	18·9	23·3*	5·2	5·3	9·1	9·7	3·9	4·4	9·8	13·6*
443	18·7	18·1	4·3	4·3	9·5	9·0	5·2	4·8	9·2	9·1
144	16·5	—	4·4	—	8·3	—	3·9	—	8·3	—
Mean	18·4	18·7	4·4	4·1	9·0	8·7	4·7	4·7	9·4	10·1
Range	16·5–20·0	17·0–20·5	3·3–5·4	2·8–5·4	7·7–10·5	6·9–10·4	3·8–5·9	3·5–6·0	8·3–10·8	8·5–11·3
S.D.	1·06	0·91	0·68	0·72	0·96	1·00	0·62	0·82	0·69	0·65
C. of V.	5·8	4·8	15·4	17·8	10·6	11·5	13·2	17·6	7·3	6·4
S.E.	0·25	0·21	0·15	0·16	0·22	0·22	0·14	0·18	0·16	0·15

* There is the possibility that this result is due to error in measurement. In any case it is excluded by Chauvenet's criterion as expounded by Davenport (1904).

TABLE 2.

Concentration and Distribution of Phosphorus in the Blood of Wethers on Pasture at The Waite Institute, Glen Osmond, South Australia.

Dropped in the North-East of South Australia towards the end of March, 1932, weaned September, 1932, received at the Waite Institute beginning March, 1933, and since then maintained on the same pasture as the sheep in Table 1.

Sheep.	Total phosphorus.		Inorganic phosphorus.		Total acid-soluble phosphorus.		Organic acid-soluble phosphorus.		Organic acid-insoluble phosphorus.	
No.	18/4/33	4/7/33	18/4/33	4/7/33	18/4/33	4/7/33	18/4/33	4/7/33	18/4/33	4/7/33
204	16·5	16·9	4·3	4·4	8·0	7·8	3·7	3·4	8·5	9·1
210	18·4	18·9	4·2	4·5	9·8	9·5	5·5	5·0	8·6	9·3
228	18·4	17·6	4·9	4·5	9·4	8·9	4·5	4·4	9·0	8·7
246	16·9	17·5	4·2	5·0	8·9	9·6	4·7	4·6	8·1	8·0

TABLE 2 (CONTINUED).

Sheep. No.	Total phosphorus. 18/4/33	4/7/33	Inorganic phosphorus. 18/4/33	4/7/33	Total acid-soluble phosphorus. 18/4/33	4/7/33	Organic acid-soluble phosphorus. 18/4/33	4/7/33	Organic acid-insoluble phosphorus. 18/4/33	4/7/33
265	19·1	18·2	5·0	5·1	8·7	8·9	3·7	3·8	10·4	9·3
275	19·8	18·9	5·1	4·5	10·9	10·0	5·8	5·4	8·9	8·9
282	18·4	19·0	4·8	4·4	9·8	9·8	5·0	5·4	8·6	9·2
283	18·4	20·8	5·5	5·9	10·2	11·3	4·7	5·4	8·2	9·5
308	17·8	18·4	4·1	4·0	9·9	9·1	5·8	5·1	8·0	9·3
315	17·6	18·0	4·0	4·3	8·9	9·1	4·9	4·8	8·7	8·9
323	—	17·4	5·0	4·0	9·6	8·6	4·6	4·6	—	8·8
330	19·4	19·3	4·8	4·9	10·0	10·5	5·2	5·5	9·4	8·3
341	19·6	18·8	5·0	5·1	10·3	9·6	5·3	4·5	9·3	9·2
343	18·4	18·8	5·1	5·3	10·2	10·1	5·2	4·8	8·1	8·7
367	19·7	19·4	4·9	4·7	10·3	10·3	5·3	5·7	9·5	9·0
375	19·6	19·7	4·9	4·8	10·2	10·4	5·3	5·6	9·4	9·3
384	19·0	19·9	4·7	4·8	9·9	10·1	5·2	5·3	9·1	9·8
385	18·2	18·4	4·6	5·0	9·4	9·6	4·7	4·6	8·8	8·8
414	18·3	17·3	5·1	4·6	9·3	8·8	4·2	4·2	9·0	8·4
416	19·1	19·7	4·3	4·6	9·4	10·2	5·1	5·6	9·7	9·5
Mean	18·5	18·6	4·7	4·7	9·6	9·6	4·9	4·9	8·8	9·0
Range	16·5–19·8	16·9–20·8	4·0–5·5	4·0–5·9	8·0–10·9	7·8–11·3	3·7–5·8	3·4–5·7	8·0–10·4	8·0–9·8
S.D.	0·89	0·98	0·39	0·44	0·66	0·77	0·57	0·61	0·60	0·40
C. of V.	4·8	5·2	8·4	9·1	6·8	8·1	11·5	12·4	6·8	4·4
S.E.	0·20	0·22	0·09	0·10	0·15	0·17	0·13	0·14	0·14	0·09

TABLE 3.

Concentration and Distribution of Phosphorus in the Blood of Wethers and Ewes on Pasture at The Waite Institute, Glen Osmond, South Australia, 31st August, 1932.

A mixed lot of variable age and condition used entirely for general scavenging.

	Total phosphorus.	Inorganic phosphorus.	Total acid-soluble phosphorus.	Organic acid-soluble phosphorus.	Organic acid-insoluble phosphorus.
1	18·8	4·8	9·4	4·3	9·4
2	15·7	4·2	8·9	4·7	6·9
3	16·6	4·4	8·0	3·7	8·6
4	18·3	4·5	9·5	5·0	8·8
5	16·7	4·5	8·5	4·0	8·2
6	19·0	5·2	9·7	4·6	9·2
7	20·1	5·2	9·7	4·6	10·4
8	18·7	4·3	8·6	4·3	10·1
9	16·5	5·2	8·7	3·5	7·8
10	—	3·9	8·3	4·4	—
11	16·6	4·2	8·3	4·1	8·3
12	—	4·4	8·5	4·1	—
13	17·4	5·1	8·3	3·2	9·1
14	14·4	3·8	7·2	3·3	7·2
15	18·6	4·4	9·2	4·8	9·4
16	18·3	6·2	11·2	5·0	7·1
17*	18·0	3·3	8·3	5·0	9·7
18	17·0	5·0	8·8	3·8	8·2
19	17·4	4·1	8·5	4·5	8·8
20	—	4·8	9·5	4·7	—
21	18·1	4·1	9·1	5·4	8·6
22	19·4	4·9	10·3	5·4	9·0

TABLE 3 (CONTINUED).

	Total phosphorus.	Inorganic phosphorus.	Total acid-soluble phosphorus.	Organic acid-soluble phosphorus.	Organic acid-insoluble phosphorus.
23	17·1	5·1	9·1	4·0	8·0
24*	18·9	4·8	9·2	4·4	9·7
25	17·9	4·8	8·7	4·0	9·2
Mean	17·7	4·7	9·0	4·4	8·7
Range	14·4–20·1	3·3–6·2	7·2–11·2	3·2–5·4	6·9–10·4
S.D.	1·29	0·59	0·83	0·58	0·92
C. of V.	9·2	12·6	9·2	13·2	10·6
S.E.	0·28	0·12	0·17	0·12	0·20

TABLE 4.

Concentration and Distribution of Phosphorus in the Blood of Sheep on Pasture at The Waite Institute, Glen Osmond, South Australia.

Summary of Tables 1, 2, and 3.

		Sheep Date	of Table 1. 3/10/32	of Table 2. 4/1/33	of Table 2. 18/4/33	of Table 2. 4/7/33	of Table 3. 31/8/32
Total phosphorus.	No. of sheep		18	20	19	20	22
	Mean		18·4	18·7	18·5	18·6	17·7
	Range		16·5–20·0	17·0–20·5	16·5–19·8	16·8–20·8	14·4–20·1
	S.D.		1·06	0·91	0·89	0·98	1·29
Inorganic phosphorus.	No. of sheep		20	20	20	20	25
	Mean		4·4	4·1	4·7	4·7	4·7
	Range		3·3–5·4	2·8–5·4	4·0–5·5	4·0–5·9	3·3–6·2
	S.D.		0·68	0·72	0·39	0·44	0·59
Total acid-soluble phosphorus.	No. of sheep		20	20	20	20	25
	Mean		9·0	8·7	9·6	9·6	9·0
	Range		7·7–10·5	6·9–10·4	8·0–10·9	7·8–11·3	7·2–11·2
	S.D.		0·96	1·00	0·66	0·77	0·83
Organic acid-soluble phosphorus.	No. of sheep		20	20	20	20	25
	Mean		4·7	4·7	4·9	4·9	4·4
	Range		3·8–5·9	3·5–6·0	3·7–5·8	3·4–5·7	3·2–5·4
	S.D.		0·62	0·82	0·57	0·61	0·58
Organic acid-insoluble phosphorus.	No. of sheep		18	20	19	20	22
	Mean		9·4	10·1	8·8	9·0	8·7
	Range		8·3–10·8	8·5–11·3	8·0–10·4	8·0–9·8	6·9–10·4
	S.D.		0·69	0·65	0·60	0·40	0·92

TABLE 5.

Concentration and Distribution of Phosphorus in the Blood of Sheep on Pasture in the Mid-North and North-East Districts of South Australia.

	Location.	North-East, South Australia.			Mid-North, South Australia.		
	Pasture.	Poor "stand" of saltbush.	Good "stand" of saltbush.	*Stipa nitida*.	Natural pasture.	Natural pasture.	Top-dressed pasture.
	Sheep.	Wether hoggets to 4-tooth.	Wether hoggets.	Ewes with 6-week lambs.	Weaners.	Ewes with lambs.	Ewes with lambs.
	No. of sheep	11	7	7	4	5	9
Total phosphorus.	Mean	17·8	17·9	17·2	17·8	16·9	18·6
	Range	15·6–19·0	16·5–20·1	14·4–19·1	16·6–18·9	16·4–17·8	17·4–20·6
	S.D.	1·16	1·19	1·61	0·84	0·56	0·98

TABLE 5 (CONTINUED).

Location.		North-East, South Australia.			Mid-North, South Australia.		
Pasture.		Poor "stand" of saltbush.	Good "stand" of saltbush.	*Stipa nitida.*	Natural pasture.	Natural pasture.	Top-dressed pasture.
Sheep.		Wether hoggets to 4-tooth.	Wether hoggets.	Ewes with 6-week lambs.	Weaners.	Ewes with lambs.	Ewes with lambs.
	No. of sheep	13	10	8	8	8	12
Inorganic phosphorus.	Mean	4·8	3·8	3·1	4·8	3·8	4·7
	Range	3·3–6·2	2·3–5·9	1·8–4·2	3·9–5·7	3·3–4·9	3·4–6·7
	S.D.	0·83	0·88	0·65	0·54	0·48	0·92
Total acid-soluble phosphorus.	No. of sheep	13	10	8	9	7	12
	Mean	9·2	9·1	7·8	8·2	7·9	9·5
	Range	6·4–10·8	8·1–11·0	4·9–9·6	7·2–9·2	7·5–8·8	7·3–11·2
	S.D.	1·37	0·88	1·56	0·59	0·46	1·06
Organic acid-soluble phosphorus.	No. of sheep	13	10	8	8	7	12
	Mean	4·4	5·3*	4·7*	3·3	4·0	4·8
	Range	2·6–5·6	4·1–6·3	3·1–6·2	2·4–4·6	3·4–4·7	3·9–5·8
	S.D.	0·88	0·64	1·12	0·68	0·37	0·53
Organic acid-insoluble phosphorus.	No. of sheep	11	7	7	4	5	9
	Mean	8·4	8·9	8·9	9·2	9·0	9·0
	Range	7·7–9·0	7·9–10·2	7·8–9·7	7·4–10·4	7·7–10·3	8·1–10·1
	S.D.	0·46	0·69	0·62	1·14	0·83	0·63

* In these observations filtration was not performed until about one hour after precipitation.

DISCUSSION.

The concentration and distribution of phosphorus in blood is rather variable. In the blood of sheep on the one pasture at the Waite Institute (Tables 1 and 2) from 16·5–20·8 mg. of phosphorus per 100 c.c. of blood were present, of which 2·8–5·9 mg. were inorganic, 3·4–6·0 mg. were organic-soluble, and 8·0–11·3 mg. were organic acid-insoluble. Thus it seems more correct to speak of normal ranges of concentration of phosphorus and its fractions in the blood than of normal concentrations.

On this one pasture the *mean* concentration of inorganic phosphorus, also, varied between 4·1 and 4·7 mg. of P/100 c.c. of blood in different series of observations, while the *mean* concentration of organic acid-insoluble phosphorus was still more variable. The available data concerning these observations is not sufficient to trace the factors governing the changes in organic acid-insoluble phosphorus, but the variation in concentration of inorganic phosphorus may be determined by the seasonal variation of phosphorus in the pasture. Unfortunately, the sheep on which the observations were made in April and July, 1933, were not of the same group as those on which the observations of October, 1932, and January, 1933, were made. Furthermore, in April, 1933, the former were but one year old, and had only been on this pasture for one month. Thus no definite conclusions can be drawn.

The observations in the Mid-North and North-East indicate variation that occurs among "normal" sheep on different pastures. The inorganic phosphorus

fraction of the ewes on spear grass in the North-East—where bone-chewing was reported to occur—the organic acid-soluble phosphorus fraction of the weaners in the Mid-North, and the inorganic phosphorus and organic acid-soluble phosphorus fractions of the ewes on natural pasture in the Mid-North exhibit concentrations significantly lower than those found at the Waite Institute, but the other figures are much the same.

SUMMARY.

In the course of 80 observations on 40 sheep on natural pasture in the Adelaide district of South Australia, from 16·5–20·8 mg., mean 18·5 mg., of phosphorus per 100 c.c. of blood were found to be present. Of this 2·8–5·9 mg., mean 4·5 mg., were inorganic, 3·4–6·0 mg., mean 4·8 mg., organic acid-soluble, and 8·0–11·3 mg., mean 9·3 mg., organic acid-insoluble. With other sheep on other pastures a somewhat wider range of values was found.

ACKNOWLEDGMENTS.

I am indebted to the Waite Agricultural Research Institute and to various station-owners of the Mid-North and North-East districts for so kindly affording me the opportunity of making observations on their sheep. My thanks are also due to Messrs. H. R. Marston, E. W. Lines, and A. W. Peirce for their invaluable assistance in the collection of the blood samples, and I would again like to express my appreciation of the hospitality and financial assistance extended to me by the Commonwealth Council for Scientific and Industrial Research.

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THE STAINING OF VITAMIN C IN THE ADRENAL GLANDS

by

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INTRODUCTION.

Szent-Györgyi (1928) found that the adrenal gland cortex contained a powerful reducing substance which possessed the ability to reduce alkaline or neutral silver nitrate solution. The adrenal cortex extracts possessed eight to twenty times the reducing power of extracts of any other tissue in the body.

A strongly reducing substance has also been obtained from lemon juice, and has been identified with vitamin C. It has been shown that it is the presence of vitamin C which gives to the adrenal cortex its reducing power.

Vitamin C has been identified as a member of the group of hexuronic acids, namely ascorbic acid, and the molecular structure, general chemical properties, etc., have been established.

The blackening of the cortex when large pieces of the gland are placed in silver nitrate solution serves as a rough test for avitaminosis C, for in experimental scurvy of guinea pigs, Moore and Ray (1932) have found the adrenals to be completely unaffected by the silver nitrate solution. They urge caution, however, in accepting lack of staining capacity of the adrenal as a conclusive test that the animal is physiologically lacking in vitamin C.

TECHNIQUE OF FIXATION.

The author has attempted by a number of techniques to demonstrate vitamin C in wax sections of the adrenal.

A non-aqueous, non-alcoholic fixative is necessary to preserve the form of the vitamin, which is soluble in alcohol and water; and the substance used must be one which will not destroy the reducing power of the vitamin. Since it was decided that formaldehyde would not destroy that portion of the molecular formation of the vitamin C which is responsible for its reducing power, it was decided to use formaldehyde vapour. Merely suspending the gland above aqueous

solutions of formaldehyde did not give good results, so the gland was accordingly suspended over heated paraformaldehyde, which gave off formaldehyde in a vapourous form.

The tissue was placed in a cradle composed of a short length of glass tube about half an inch in both length and diameter, over the bottom of which was fastened a small piece of cheese cloth. The cradle was supported by two enamel covered wires. About 3 to 5 gm. of pure paraformaldehyde were placed in the bottom of a large test tube. The cradle was lowered to within an inch of the paraformaldehyde and the carrying wires projecting beyond the end of the tube were bent over, and the opening loosely closed with a cork. The tube was then heated gently until the paraformaldehyde commenced to decompose, and the heating was continued cautiously until the reaction was complete. The tissue was permitted to remain in this vapour for a further ten to fifteen minutes, and was then removed and cut into small pieces. Where more efficient penetration of the fixative was required, the heating was stopped after approximately half the paraformaldehyde had decomposed, and the tissue was then removed, cut into thin pieces, and returned to the cradle for a further period.

Two other methods of fixation were attempted. Fixation in 70 p.c. alcohol to which silver nitrate had been added so that reduction and fixation took place simultaneously, and fixation by means of chloroform with or without silver nitrate in solution was also used. In none of these methods was fixation as successful as with the paraformaldehyde method. A further method of fixation was adopted using osmium tetroxide (osmic acid) in a 1·5 p.c. aqueous solution. It was thought that this might impregnate the vitamin C.

ATTEMPTED METHODS OF DEMONSTRATION OF VITAMIN C IN FIXED TISSUE.

A number of methods were used, all based on the reducing power of the vitamin, and they are enumerated below:

1. Reduction of silver nitrate in aqueous solution.
2. Reduction of silver nitrate in alcoholic solution.
3. Reduction of silver nitrate in chloroformic solution.
4. Reduction of osmium tetroxide in aqueous solution.
5. Reduction of Fehling's solution by the monoacetone derivative of the vitamin.
6. Reduction of Fehling's solution by the vitamin.
7. Reduction of acidulated gold chloride in the presence of an oxidizing substance.

The first method was one of the most successful. The second also gave good results. In the third method the gland was either fixed first in formaldehyde vapour and then added to the solution, or it was placed directly into the chloroform. In the first case both fixation and impregnation were very satisfactory; in the second case, while impregnation was good, fixation was poor.

Where paraformaldehyde fixation was adopted, the gland tissue on fixation was cut into two, washed for a few seconds in distilled water, and then dropped into the silver nitrate solution. In some cases fresh glands were placed directly into alcoholic silver nitrate or chloroformic silver nitrate. In all cases the period in the nitrate solution to secure adequate penetration was about 12 hours. Sections of tissue impregnated with silver nitrate were treated with gold chloride and photographic hypo for toning purposes in the same manner which is adopted for Da Fano Golgi preparations.

In the fourth method a possible reaction with the adrenalin granules and cortical fat was expected; the medulla appeared comparatively clear when sections of the gland were made, and the cortex had blackened quite considerably. The typical form of the vitamin C was absent.

The fifth method, the reduction of Fehling's solution by the monoacetone derivative of the vitamin, was attempted by the following technique:

1. The gland tissue was fixed in formaldehyde vapour.
2. The tissue was cut into small pieces and dropped into acetone which contained anhydrous copper sulphate. The tissue was left for twenty-four hours in this mixture.
3. Each piece of gland tissue was then cut into two, and placed in Fehling's solution overnight. Then washed, etc., and sectioned.

The results from this method were very variable. One or two good results have been obtained, but the chief difficulty appears to be that the vitamin possesses a tendency to dissolve out into the acetone solution during the formation of the monoacetone derivative. One difficulty with both this method and the next (6th) was that the effect of the Fehling's solution on the cells was rather destructive. In the sixth method the tissue was dropped directly from fixation into Fehling's solution.

The gold chloride method was used in order to confirm the form of the material believed to be vitamin C in the silver preparations, and to ensure that the granules obtained in the silver nitrate method were not due to particles of formaldehyde remaining in the tissue, reducing the silver nitrate. Gold chloride is normally reduced by formaldehyde also, but if it has been previously acidified there is no reaction. With this reagent very good, though erratic, preparations were obtained.

Attempts to dissolve the substance believed to be vitamin C out of the gland by simply immersing small pieces in water were attempted as controls. The results were very variable. The size of the piece of tissue used was of considerable importance, and also the volume of water used, and whether it was static or running. The slow process of molecular diffusion of a substance such as the vitamin would require some hours to take place to any extent, and by this time autolytic changes have taken place in the cells.

CYTOLOGY OF THE SUBSTANCE BELIEVED TO BE VITAMIN C IN THE ADRENAL.

Adrenal glands from the following animals were examined by the above techniques: Domestic mice, albino mice, a twelve-hour-old albino rat, cats (one starved and one well fed), and guinea pigs.

In all these animals the form taken by the substance regarded as vitamin C was fairly constant. The argentophilic material occurred chiefly in the form of even granules, although in some cases, particularly where the material was not abundant, it tended to occur in the form of irregular granules, at times elongated, and simulating in many ways the typical Golgi apparatus.

In cells which contained no lipoid globules, the vitamin was scattered through the cytoplasm of the cell, but was particularly dense in the region of the nucleus, there being a distinct perinuclear aggregation, in some cases so intense that the nucleus was quite obscured. For a short distance inwards from the cell membrane the granules were very densely packed, and the numbers graded off towards the centre of the cell and then became densely packed again as the nucleus was approached.

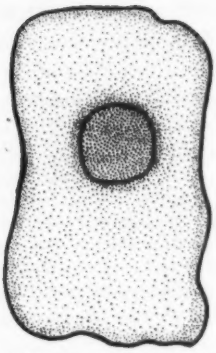

Figure 1. Diagrammatic representation of a single cortical cell to illustrate the typical disposition of the argentophile particles within the cell.

Where the cortical cells commenced to secrete lipoidal material a change in the morphology of the argentophile substance occurred. The small granules surrounding the nucleus appeared to have coalesced and formed larger and more irregular granules, which occasionally joined up to form short rods. A number of granules persisted in the region of the cell membrane, but the majority appeared to have merged into a few large granules which came into association with the developing lipoid globules, taking up various shapes in the process.

As the number of lipoid globules increased in one portion of the cell, the argentophilic particles became smaller, but each lipoidal globule had one or more small granules of the substance attached to it. At the final stage, when the cell became packed with lipoid globules, the argentophile material appeared once more to have broken up into fine granules, and each globule of lipoid was surrounded by a ring of closely packed granules. These changes in the substance believed to be vitamin C were observed in the cat and guinea pig adrenals. In one cat, which was ill-nourished, lipoid packed cells were not plentiful, and a number of stages of association of the supposed vitamin C with the lipoid granules as described were found, the argentophile material only occurring in a narrow band in the outer

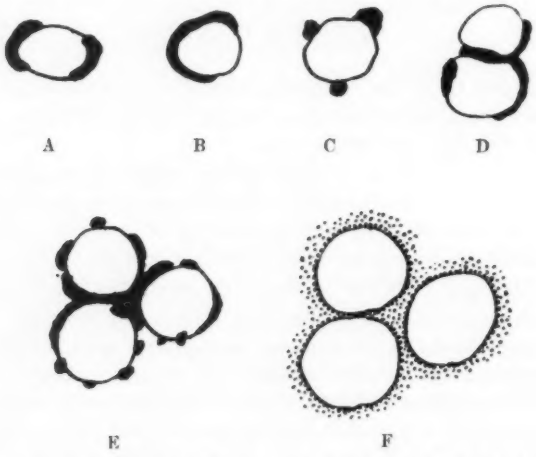

Figure 2. Showing the form occupied by the argentophile material in association with the lipoid granules of the cortex.

portion of the zona fasciculata. In another cat, which was well nourished, practically all the cells of the zona fasciculata were packed with lipoid globules, with the argentophilic substance occupying the position previously described. Some of this material was also present in the cells of the zona glomerulosa, but appeared to be absent from the cells of the inner portion of the zona fasciculata and the whole of the zona reticularis.

In a healthy guinea pig all stages were found, from cells containing no lipoid globules to cells containing, one, two, three, or a dozen globules up to cells completely packed with them.

NUCLEAR RELATIONS OF VITAMIN C.

The nuclear relations of the supposed vitamin are illustrated in the figures. The nucleus may be completely covered with fine regularly-sized granules, or there may be aggregations of large granules at one pole of the nucleus with finer granules at the opposite pole. There may be a few large regularly shaped granules or a few large but very irregular granules. At times the granules appear to merge into masses of argentophile material. They may at times form branches which are strikingly reminiscent of the Golgi apparatus.

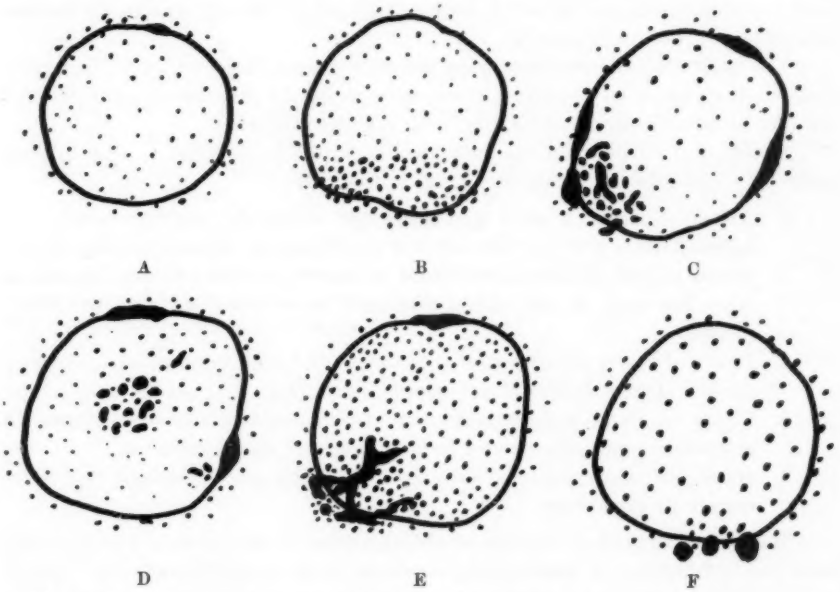

Figure 3. Different nuclei showing the variations of form assumed by the argentophile material.

This aggregation of the material at nuclear and cell membranes and also around the lipoid globules may have some significance, but the position is quite adequately explained on the assumption of purely physical forces such as surface tension, this force also tending to cause aggregation of substances such as the Golgi apparatus in similar sites.

CORTEX AND MEDULLA.

Szent-Györgyi (1928) states that the cortex of ox adrenals reacts strongly with silver nitrate, but the medulla does not. Other workers were not able to find such a distinction. In the cat the present author found that the medulla at times did not react to silver nitrate solution, but the medulla in both the mouse and the guinea pig did so, although in some cases they did not react until some time after the cortex had blackened. In a later paper evidence will be presented which shows that the concentration of the vitamin in the medulla varies under certain physiological conditions.

DISCUSSION.

There is a distinct possibility that some of the granules regarded as vitamin C may be artefacts, or that other substances such as mitochondria, Golgi material, and free cell lipoid, all of which may reduce silver nitrate, are being demonstrated in addition to vitamin C.

The possibility of artefacts being present, due to the reaction of formaldehyde in the tissues with silver nitrate, was, as before mentioned, overcome by conducting a duplicate impregnation with acid gold chloride.

Differentiation between the substance regarded as vitamin C and lipoidal structures was obtained by the following procedures:

1. Alcoholic silver nitrate was used for duplicate impregnations with aqueous silver nitrate. The alcohol would tend to disperse the lipoids.
2. Pieces of gland tissue were fixed in paraformaldehyde and soaked in ether for some hours. They still gave good reactions with the silver nitrate.
3. Pieces of gland were fixed in chloroform and then impregnated, and still showed large numbers of argentophile granules.
4. Pieces of gland were impregnated with a solution of silver nitrate in chloroform, and also showed large numbers of black granules.
5. Duplicate impregnations with acid gold chloride confirmed the silver nitrate preparations.

Harris has suggested that the chemical nature of the vitamin makes it possible that it functions in maintaining adrenalin in its reduced condition. This is quite a likely explanation where the vitamin is present in the medulla, but is rather difficult of application where it is absent.

American workers have found that if cortin, prepared so as to contain no vitamin C, were given to guinea pigs on a scorbutic diet, the onset of scurvy was delayed. From this result it was suggested that cortin is concerned in the utilization of vitamin C. The point which becomes apparent here is, however, that vitamin C, from its association with the lipoid globules of the cortex, which are probably the precursors of cortin, may have some function in the synthesis of this hormone; and that cortin may be thus more directly concerned in the condition of scurvy.

SUMMARY.

A substance has been demonstrated in the cortex and medulla of the adrenal glands of certain mammals which will, unaided, reduce silver nitrate from aqueous, alcoholic, and chloroformic solutions, producing discrete black granules scattered through the cell, with a tendency towards perinuclear aggregation, and a tendency to aggregate around the lipoidal granules of the adrenal cortical cell. It will reduce acidulated gold chloride in aqueous solution, and is distinct from the lipoidal elements of the cell.

It is possible that this substance, from the similarity of its reactions with ascorbic acid, may represent the actual form and position of vitamin C in the cells of the adrenal gland.

ACKNOWLEDGMENT.

I am indebted to Professor G. Tattersall and Dr. G. A. Elliott, of the Chemistry Department of this University, for generous assistance in some of the chemical problems which it was necessary to overcome in conducting this work.

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THE DISTRIBUTION OF WATER IN LIVING TISSUES

by

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INTRODUCTION.

Various conceptions as to the manner of partition of water in the organism are to be found in the literature on water metabolism, Achard (1922), Fischer (1921), Hill (1930), Krevisky (1930), Thoenes (1925), Siebeck (1926); but three fundamental types of distribution are widely recognized: chemically bound water, water "bound" by tissue colloids, also known as water of imbibition or swelling water, and "free" water or reserve water. Of the numerous investigations into the regulation of the water distribution some results point to a central nervous control, others to a hormone control, and yet others to a simple physico-chemical control. The present work is based upon the method of Andrews (1926), who investigated the diuresis produced in dogs, by the continuous intravenous injection of hypertonic glucose or saline solutions. The extent of the diuresis depended upon the "reserve water" of the body, i.e., the amount of "free" or loosely bound water which could be withdrawn by this method from the body tissues. Andrews further showed that the quantity of reserve water was a function of the tissue reaction as measured by the alkali reserve of the blood; the more alkaline the tissues the greater the reserve water, and *vice versa*. Although Andrews' results show a remarkable regularity, in the absence of experimental details it is difficult to understand how biological variations were so successfully controlled.

Rabbits were used in the present work. Simultaneous determinations of tissue water, blood dilution, and other relevant estimations have been carried out in an attempt to ascertain the source of the reserve water.

EXPERIMENTAL PROCEDURE.

Throughout the series of experiments male rabbits alone were used in order to avoid effects of the sexual cycle on the water metabolism. The work was divided into two series of experiments, as follows:

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[1] John L. Young Scholar.

Series I.

This comprised fourteen separate experiments, using rabbits whose previous dietary history was either not known or not controlled, except that in a period varying from 15 to 36 hours, just prior to an experiment, the animals were deprived of food and water. The period of starvation was varied in length so as to try and bring about wide variations in the plasma CO_2 capacity of the animals. The degree of acidosis induced, however, bore no relationship to this period of starvation. In some of the animals extreme acidosis was brought about by the peroral administration of either 5 p.c. ammonium chloride solution (5 c.c. per kg. of body weight) or 2 p.c. hydrochloric acid (7 c.c. per kg. of body weight). The administration was carried out immediately prior to the starvation period.

Series II.

This comprised six separate experiments, using animals whose previous dietary history was controlled. These rabbits, for a week prior to an experiment, were fed on a mixture of dried lucerne and wheat, with 90 c.c. of water per diem (this quantity of water was found by trial to be about the normal amount required by rabbits on the above diet in winter months). For the last 16 hours the animals were deprived of all food and water. In order to precipitate an acidosis in some of these animals the 5 p.c. intravenous saline was made N/100 or N/10 acid by addition of lactic acid or N/20 acid by means of hydrochloric acid.

Method of Injection.

The injections were carried out with a modified form of the Woodyatt (1917 and 1920) continuous intravenous injection pump, which was standardized to deliver 25 c.c. per hour, with an accuracy of 2 p.c. As in Andrews' experiments with dogs, the fluid injected was 5 p.c. pure sodium chloride solution at the constant rate of 25 c.c. per kg. of body weight per hour for a period of 90 minutes. The saline after leaving the syringes of the pump passed through a small reservoir heated to such a temperature that it was delivered at the cannula at blood temperature. No precautions as to sterility were taken, as all animals were destroyed at the end of each experiment.

Operative Procedure.

All rabbits were given, by means of a stomach tube, 1·6 c.c. of paraldehyde per kg. of body weight (to the nearest 10 gm.). About one hour later, when narcosis was almost complete, the neck incision was made, some 5 c.c. of ether being necessary, in most instances, to deepen the anaesthesia.

By means of a well-paraffined hypodermic syringe, 5 c.c. of blood were withdrawn from one of the external jugular veins (for haemocrit, plasma CO_2, osmotic pressure, and blood dilution estimations). In order to measure the urinary output, the bladder was catheterized with a rubber catheter, which was tied in. All the urine was then expressed from the bladder and rejected.

The next step was to insert the infusion cannula into the external jugular vein. The time of commencement of injection was noted and injections were stopped 90 minutes later. Immediately after insertion of the cannula, two samples of pectoral muscle (on the same side) were taken for the determination of tissue water. The neck wound was then closed with clips. As a rule diuresis set in about 15 minutes after the beginning of the injection, and reached a maximum about one hour later. Even after stopping the injection at the end of 90 minutes, diuresis still continued, at diminishing rate, for a further 30 to 60 minutes. The average duration of an experiment was thus 2½ to 3 hours. When diuresis had ceased, the total volume of urine passed by the catheter was noted, and further samples of blood and pectoral muscle (from the opposite side) were taken for analysis.

Throughout both series of experiments all animals withstood the operative procedure without the least indication of disturbance other than a slight fall in respiratory rate as the experiment proceeded.

METHODS OF ANALYSIS.

Water Loss.

This was determined by measuring the total urinary output to the nearest c.c. and subtracting from this figure the amount of saline injected. The result is expressed as c.c. of water lost per kilo. of body weight. In some experiments the urinary output was less than the intake of saline, and the difference in these cases therefore represented a water retention.

Plasma CO_2 Capacity (Alkali Reserve).

Duplicate analyses were made by the method of Van Slyke and Neill (1924), the method for micro-estimations on 0·2 c.c. plasma being used. The degree of accuracy of this determination was such that individual figures did not vary from the average of duplicate analyses by more than 1·5 p.c. All plasma CO_2 capacities are corrected to read—vols. CO_2 per 100 c.c. of plasma saturated with CO_2 at 40 mm. pressure and at 20°C. Clotting was prevented by the addition of approximately 0·1 p.c. of Merck's neutral potassium oxalate.

Blood Dilution.

This was checked by the simultaneous estimation of blood iron and corpuscular volume.

The blood iron estimation was carried out on duplicate samples (0·2 c.c.) of whole blood by the method of Smirk (1927), which depends upon the oxidation of all organic matter by means of concentrated nitric acid and ammonium persulphate, with the subsequent colorimetric estimation of iron as ferric thiocyanate. In our experience the above oxidizing mixture did not remove all the pigment of the blood, and this interfered with the subsequent colour-matching. This was overcome by subsequent oxidation with $KMnO_4$ or H_2O_2 (Cox and Hicks, 1933). Individual blood iron values varied from the mean values by about one p.c.

The corpuscular volume was satisfactorily determined by the micro-haematocrit method of Smirk (1928). The mean values have an accuracy of the order of 1·5 p.c.

Blood Chloride.

This was estimated by the micro-method of Smirk (1927), using duplicate 0·2 c.c. samples of whole blood. The degree of accuracy is one p.c.

Tissue Water.

Duplicate samples of pectoral muscle weighing about 0·2 gm. were placed in tared porcelain crucibles in a covered vessel until weighed, and were then dried in a steam oven to constant weight. The difference in water lost was taken to represent water gained or lost from the muscle during the experiment. Mean values have an accuracy of one p.c.

Urinary Chloride.

The Volhard method was used, duplicate samples being withdrawn from the mixed total volume of urine excreted. The new Hellige immersion colorimeter[2], which allows for the "own colour" of a solution to be compensated on the Walpole principle, was employed. This estimation was carried out only in a few experiments, and generally on samples of urine passed during each three-quarters of an hour. Since comparative pH values only were

[2] F. Hellige & Co., Freiburg in Breisgau.

required, no precautions were taken to prevent contact of the urine with the atmosphere. In cases where the urine was very turbid it was filtered before testing.

Osmotic Pressure of Serum Colloids.

The method of Krogh (1927) was used, 1·5 c.c. of blood being required for the preparation of sufficient serum. The clot was separated by 20 minutes' incubation at 20°C., followed by cooling to 0°C.

The plasma CO_2, blood dilution, blood chloride, tissue water, and osmotic pressure estimations were performed on samples taken before commencement of saline infusion and after the cessation of diuresis.

RESULTS.

Alkali Reserve and Water Loss.

Series I.

The corresponding values for these two quantities are set out in Table 1.

TABLE 1.

Expt. No.	8	15	9	11	10	14	7	4	12	5	18	17	13	16
Plasma CO_2 before injection	39·0	43·7	45·5	42·6	48·0	47·4	46·7	44·9	50·6	56·5	55·7	66·9	74·9	49·8
Plasma CO_2 after Diuresis	40·3	37·8	41·7	45·1	44·5	45·9	52·4	54·3	—	48·7	53·6	49·0	62·1	47·5
Mean Plasma CO_2	39·7	40·8	43·6	43·9	46·3	46·7	49·6	49·6	50·6	52·6	54·7	58·0	68·5	48·7
Water loss	—	6	62	11	51	12	26	31	—	—	43	—	46	Blank
Water retained	3								5	20		8		

TABLE 1A.

Before Injection.

$n=20$, $\Sigma x=1049$, $M_1=\frac{\Sigma x}{n}=52{\cdot}5$

$\Sigma\Delta x^2=2154{\cdot}76$

$\mu=\pm\sqrt{\frac{\Sigma\Delta x^2}{n-1}}=\pm 10{\cdot}65$

$\mu_{x_1}=\pm\frac{\mu}{\sqrt{n}}=\pm 2{\cdot}4$

After Cessation of Diuresis.

$n=18$, $\Sigma x=867$, $M_2=\frac{\Sigma x}{n}=48{\cdot}2$

$\Sigma\Delta x^2=884{\cdot}51$

$\mu=\pm\sqrt{\frac{\Sigma\Delta x^2}{n-1}}=\pm 7{\cdot}21$

$\mu_{x_2}=\frac{\mu}{\sqrt{n}}=\pm 1{\cdot}7$

$$k=\frac{M_1-M_2}{\sqrt{\mu^2_{x_1}+\mu^2_{x_2}}}=\frac{52{\cdot}5-48{\cdot}2}{\sqrt{(2{\cdot}4^2+1{\cdot}7^2)}}=\frac{4{\cdot}3}{2{\cdot}9}>1<2,$$

Agreement between the two series of plasma CO_2 determinations is satisfactory.

It will be seen from the above table that in every instance the plasma CO_2 capacity altered during the course of an experiment, and so the mean of the values before and after the experiment was chosen as the quantity to be correlated with the water loss. The alteration in plasma CO_2 capacity (generally a fall) was not inconsiderable, in fact much greater than the experimental error of 1·5 p.c. On the other hand, statistical analysis of the data given in Table 1a, according to Pütter (1929) showed no significant difference between the two series of plasma CO_2 capacities. Conclusions from the application of the statistical method to such a small series must be taken with reserve; hence, the difference between the plasma CO_2 values must be considered to be a real one.

Other workers have reported changes in the alkali reserve of the blood under conditions similar to those of our experiments; Fujimaki (1924) observed that injection of saline into rabbits led to a rise of alkali reserve, whilst Potter (1925) records a fall of from 3 to 10 vols. p.c. in alkali reserve as an effect of anaesthesia.

The acidosis in experiments 8 and 9 was due to peroral administration, 18 hours previously, of 5 p.c. ammonium chloride solution, and in experiments 15, 10, and 18 to 2 p.c. hydrochloric acid.

Experiment 16 was carried out as a blank, i.e., the experimental procedure was the same, except that no infusion was made. From the plasma CO_2 values in this experiment, it would seem that operative procedure had no notable effect upon alkali reserve.

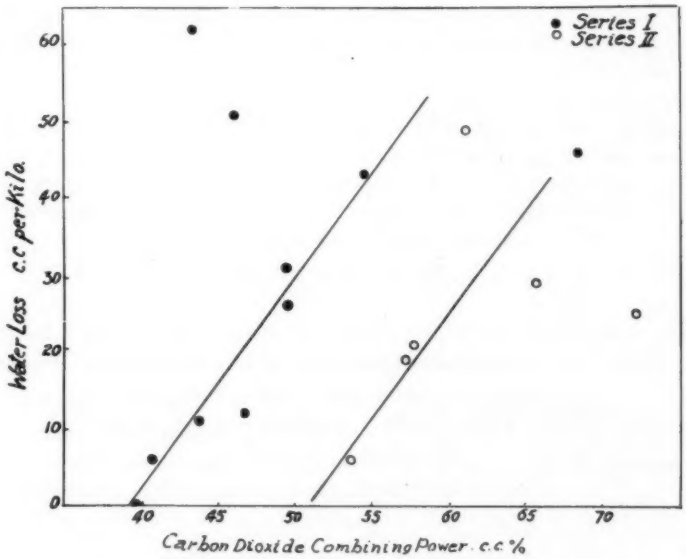

Fig. 1. Alkali reserve and water loss.

Inspection of the alkali reserve and water loss values reveals a relationship between the two, a fact made more evident when the two quantities are plotted against one another, as in fig. 1. It will be seen that some of the points (the water retention figures are plotted as zero water loss, following the convention used by Andrews) are widely scattered, but seven are distributed very closely about a straight line. Thus linear grouping of the latter seven points is the most characteristic feature of the graph, and so in the calculation of correlation and regression coefficients, the six widely aberrant values were neglected. The regression line agrees well with the plotted points, and the regression coefficient indicates that for each c.c. increase in plasma CO_2 the water loss increases by 2·9 c.c. (See Table 1b and fig. 2).

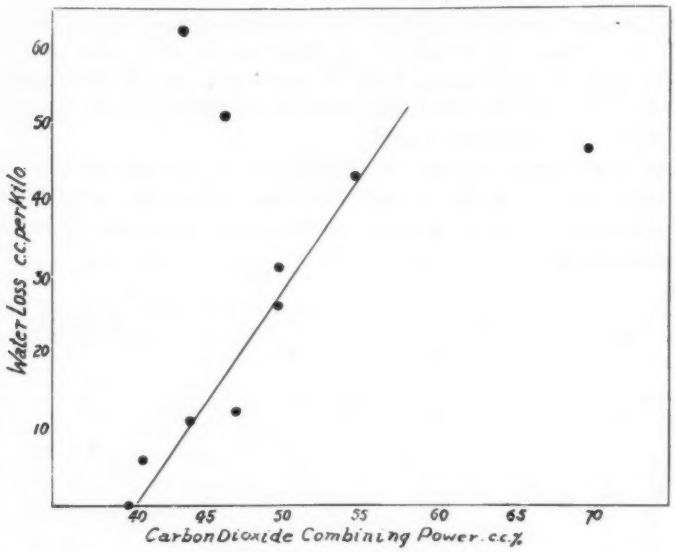

Fig. 2. Regression line.

TABLE 1B.

Δx_2 = deviation from mean of plasma CO_2.

Δx_1 = deviation from mean of water loss.

$\Sigma \Delta x_1 . \Delta x_2 = +47{\cdot}82$, $\sigma_1 = \pm 1{\cdot}53$, $\sigma_2 = \pm 5{\cdot}35$, $n = 7$

Correlation Coeff., $r_{12} = \dfrac{\Sigma \Delta x_1 . \Delta x_2}{n . \sigma_1 . \sigma_2} = 0{\cdot}834$

$$\mu_r = \pm \frac{1 - r^2}{\sqrt{n}} = \pm 0{\cdot}26$$

Regression Coeff., $b_{21} = r_{12} . \dfrac{\sigma_2}{\sigma_1} = 2{\cdot}9$

The aberrant values are difficult to explain. They may be due to the fact that the characteristically large caecum of the herbivora permits large and variable storage. As explained above, the diet in this series was not controlled.

In Series II an attempt was made to control these possible disturbing factors.

Series II.

Results are presented in the same manner as in Series I.

TABLE 2.

Expt. No.	25	20	22	23	21	24
Plasma CO_2 before injection	68·9	56·5	63·3	61·2	65·5	73·8
Plasma CO_2 after diuresis	53·7	58·0	52·8	61·2	66·2	70·7
Mean Plasma CO_2	53·7	57·2	57·8	61·2	65·8	72·2
Water loss	6	18	21	49	29	25

In experiments 23 and 24 the infusion saline was made N/100 and N/10 acid respectively with lactic acid, and in experiment 25, N/20 acid with hydrochloric acid. The plasma CO_2 values show little, if any, alteration from the introduction of lactic acid, but with the hydrochloric acid there was a large fall. Owing to this latter fact, the mean plasma CO_2 value for experiment 25 was taken to be the same as the plasma CO_2 after diuresis. Fig. 1 shows the result of plotting alkali reserve against water loss, and except for the two lactic acid infusions, a close linear relationship is evident. The fact that these curves are parallel indicates that there was no dietary alteration in the water metabolism. Owing to the small number of observations in this series, no statistical analysis was applied, but from inspection of results it appears that the same conclusions are to be drawn as in Series I.

BLOOD DILUTION.

Under this heading are grouped the results of the blood-iron and corpuscular volume estimations, which for series I experiments are shown in Table 3.

Fe_1 = blood iron (mg. per 100 c.c. blood) before saline infusion.
Fe_2 = ditto after cessation of diuresis.
V_1 = corpuscular volume prior to infusion.
V_2 = corpuscular volume after cessation of diuresis.

TABLE 3.

(Experiments in order of ascending alkali reserve.)

Expt. No.	8	15	9	11	10	14	7	4	12	5	18	17	13	16
Blood Iron														
Fe_1	48·4	60·9	62·4	54·1	62·2	61·6	44·6	49·7	52·3	46·3	54·2	50·8	59·6	58·1
Fe_2	43·6	56·0	63·5	52·9	57·5	60·0	42·6	42·7	48·5	39·1	49·9	50·0	57·9	49·9
$\frac{Fe_2}{Fe_1}$	0·901	0·920	1·018	0·978	0·925	0·974	0·955	0·859	0·927	0·845	0·921	0·984	0·972	0·859
Corpuscular Volume														
V_1	35·8	46·4	45·9	39·4	48·7	46·5	40·6	35·8	37·8	36·1	39·3	37·1	42·4	36·8
V_2	31·2	44·1	47·3	38·9	—	47·2	40·0	26·2	33·9	29·2	37·1	33·7	40·7	30·0
$\frac{V_2}{V_1}$	0·872	0·950	1·031	0·987	—	1·015	0·985	0·732	0·897	0·809	0·944	0·908	0·960	0·815

Analysis of Table 3.

Blood Iron.

$$k=\frac{3\cdot5}{2\cdot0}>1<2$$

i.e. there is satisfactory agreement between the two groups of blood-iron values.

Corpuscular Volume.

$$k=\frac{3\cdot8}{1\cdot1}>3<4$$

i.e. there is a significant difference between the two corpuscular volumes.

The difference between the values for Fe_2 and Fe_1 was of the order of 5 p.c., a difference far greater than the experimental accuracy of 1 p.c. However, statistical analysis of the data does not show this difference to be a significant one. Similarly, in the case of the corpuscular volume measurements there is an average difference of 10 p.c. (experimental accuracy 1·5 p.c.), a difference which statistically is not certainly a significant one. Taking into account the relatively small number of measurements made, the statistical results are not entirely trustworthy, but it is justifiable to conclude that the difference between our analytical results is of some significance. This being so, comparison of the ratios Fe_2/Fe_1 and V_2/V_1 will give some indication of the extent of blood dilution and gross exchange in corpuscular volume. The mean values of Fe_2/Fe_1 and V_2/V_1 for Series I experiments are found to be 0·937 and 0·924 respectively. It is inferred from these data that, at the cessation of diuresis, the blood was even then in a state of dilution, and also that the red corpuscles had suffered some shrinkage, possibly as a direct effect of the hypertonic saline entering the blood-stream. A shrinkage is also to be expected even if the blood were not hypertonic, owing to increase in plasma Cl' and the resulting shift from the corpuscles of HCO'_3 in order to restore electrolyte distribution in conformity with Donnan's law. Investigation of individual V_2/V_1 ratios revealed no correlation between water loss and corpuscular shrinkage.

Experiment 16 in the above table gives the figures for the blank experiment. The decrease in both blood-iron and corpuscular volume was here much greater than in any other experiment. A dilution of the blood due to haemorrhage (7 c.c.

of blood taken) is evidently indicated, a fact which is quite clear in Skelton's investigations (1926, 1927). Hence, the dilution observed in the rest of our experiments may have its origin in the haemorrhage due to withdrawal of the first blood sample, although the infusion of saline must also be a factor.

In Table 4 are tabulated the estimations of blood-iron and corpuscular volume for Series II experiments. The same remarks apply to these results as to those in Table 3.

TABLE 4.

Expt. No.		25	20	22	23	21	24
Blood Iron	Fe_1	54·1	61·7	56·2	59·8	60·7	50·8
	Fe_2	50·7	56·0	55·9	55·4	54·3	50·6
	Fe_2/Fe_1	0·937	0·908	0·995	0·927	0·895	0·994
Corpuscular Volume	V_1	41·9	44·3	40·7	42·2	49·7	36·6
	V_2	37·8	39·0	38·3	39·8	40·8	36·3
	V_2/V_1	0·902	0·881	0·941	0·943	0·821	0·992

In both Tables 3 and 4, fluctuations of the blood-iron values (Fe_1) from animal to animal are closely parallel to similar fluctuations in corpuscular volume values (V_1). This indicates that all the erythrocytes possessed a constant haemoglobin concentration.

TISSUE WATER.

The tissue water values for the experiments in both Series I and II are given in Tables 5 and 6 respectively, and are expressed as grams of water per 100 gm. of pectoral muscle. The difference between tissue water before injection of saline and after diuresis indicates the extent of dehydration or hydration of muscle.

TABLE 5.

Expt. No.	8	15	9	11	10	14	7	4	12	5	18	17	13	16
Tissue water before injection	—	71·2	70·0	71·9	70·4	72·5	67·2	69·3	73·9	72·7	74·2	71·9	70·7	74·8
Tissue water after Diuresis	—	71·6	70·3	72·2	71·5	73·0	69·7	68·2	72·4	70·2	69·7	74·0	69·1	76·7
Dehydration of muscle	—							1·1	1·5	2·5	4·5		1·6	
Hydration of muscle		0·4	0·3	0·3	1·1	0·5	2·5					2·1		1·9
Body water loss	+3	6	62	11	51	12	26	31	+5	+20	43	+8	46	—

TABLE 6.

Expt. No.	25	20	22	23	21	24
Tissue water before injection	72·1	71·6	72·5	71·9	71·9	71·7
Tissue water after diuresis	70·4	68·4	70·1	68·9	67·1	71·0
Dehydration of muscle	1·7	3·2	2·4	3·0	4·8	0·7
Body water loss	6	18	21	49	29	25
Computed water loss	9	17	13	16	25	4

Water balance values are included in the above tables for comparison purposes. The experimental error of this estimation was of the order of 1 p.c., from which it follows that many of the hydration values of muscle (Table 5) can have no significance because they lie within the limits of experimental error. In both series the dehydration figures lie outside this limit, and are taken to indicate a significant dehydration of the muscle.

Muscle hydration occurred only in the Series I experiments, and this even in cases where the body water loss was very considerable. Moreover, muscle dehydration occurred not only when there was body water loss, but also when there was water retention (e.g., experiments 12 and 5). No correlation could be discovered between the loss of water from the tissues and from the body as a whole.

In experiment 16 (blank) there was an hydration of muscle tissue, an unexpected result, because generally haemorrhage induces tissue dehydration (Skelton), although in the experiments of the latter the haemorrhage was more severe.

In Series II muscle dehydration was observed in all experiments (see Table 6), this being in agreement with the findings of Tashiro (1926) and of Skelton. The latter, who examined the effects of saline infusions upon the various tissues of the cat, observed that following upon isotonic and hypertonic saline infusion, all tissues, except muscles, showed an increase in water content, whereas hypertonic saline dehydrated muscle tissue. These facts are attributed to the non-permeability of the muscle cell to Cl'.

In these experiments (Table 6) there is a tendency for the extent of muscle dehydration to run parallel with the extent of the loss of water from the body, except in the anomalous experiments 23 and 24, in which lactic acid was injected. According to Skelton the quantity of muscle in a rabbit is 52·2 p.c. of the body weight. This has been used to compute the fraction of the total water loss that was derived from muscle tissue alone (see Table 6). The agreement between computed and observed body water loss is satisfactory, if we exclude the aberrant values in experiments 23 and 24; but even these latter are not without significance when it is considered that lactic acid is peculiarly important in muscle metabolism. The infusion of lactic acid must have disturbed the normal metabolism of the muscle and hence its water balance.

BLOOD CHLORIDE.

The chloride content of whole blood was estimated and is expressed as mg. NaCl per 100 c.c. of blood, the figures for Series I experiments being set down in Table 7.

TABLE 7.

Expt. No.	8	15	9	11	10	14	7	4	12	5	18	17	13	16
Blood chloride before injection	544	588	500	525	500	519	500	—	500	475	569	525	488	550
Blood chloride after diuresis	700	713	681	731	688	700	669	—	688	600	713	725	663	581
Blood chloride increase	156	125	181	206	188	181	169	—	188	125	144	200	175	31
Normality of Bld. Cl.														
Before injection	0·093	0·101	0·085	0·090	0·085	0·089	0·085	—	0·085	0·081	0·097	0·090	0·083	0·094
After diuresis	0·120	0·122	0·116	0·125	0·118	0·120	0·114	—	0·118	0·103	0·122	0·124	0·113	0·099

The experiments are set down in the order of ascending alkali reserve values.

Analysis of Table 7 shows that $k=\frac{170}{4}=12{\cdot}1$ i.e. >5.

There is therefore a significant difference between the two series.

Since the degree of accuracy in this estimation was of the order of one p.c., it follows that the increase in blood chloride observed at the end of an experiment, and which far exceeds one p.c. of the original chloride values, must be a significant increase. Furthermore, this fact is supported by the statistical analysis.

In all experiments the chloride content of whole blood chloride increased very considerably (40 p.c. increase on the average), but unfortunately no analyses were made to determine the partition of this increased chloride between cells and plasma. The normality figures in the table refer to the normality of NaCl with respect to whole blood. Hence we cannot infer from these data that the plasma was hypertonic with respect to the corpuscles at the cessation of diuresis, for the electrolytes would be distributed so as to ensure conformity to the Donnan law, namely:

$$\frac{[HCO_3'] \text{ cells}}{[HCO_3'] \text{ plasma}} = \frac{[Cl'] \text{ cells}}{[Cl'] \text{ plasma}}$$

nor can we infer that the whole blood was hypertonic with respect to the tissues on account of these latter having taken up a very considerable amount of the infused NaCl (see later under Urinary Chloride). However, during the actual infusion of saline, there can be little doubt that the blood was hypertonic with respect to the tissues, this hypertonicity constituting the force whereby water was drawn from the tissues of the animal; but the data obtained from samples taken at the cessation of diuresis probably indicate a state of equilibrium.

No relationship is evident between the increase in blood chloride and the loss of water from the body.

In experiment 16 (blank) there was a small increase in blood chloride. Since blood dilution occurred in this experiment (see Table 3), it would appear that the diluting fluid derived from the tissues was a saline solution, a fact which agrees with recent work by Hicks and Smirk (1930). In Table 8 are shown the results for Series II experiments; the same discussion and remarks as above also apply to them.

TABLE 8.

Expt. No.	25	20	22	23	21	24
Blood chloride before injection	513	475	519	500	481	500
Blood chloride after diuresis	725	663	663	663	663	656
Blood chloride increase	212	188	144	163	182	156
Before injection	0·088	0·081	0·089	0·085	0·082	0·085
After diuresis	0·124	0·113	0·113	0·113	0·113	0·112

URINARY CHLORIDE.

The total chloride excreted, expressed as grams NaCl per kilo of body weight, was computed from the urinary analyses. Since the amount of saline injected into each animal was known, as well as the exact concentration of the saline, the amount of NaCl retained (there was always some retention) per kg. of body weight could be calculated. Tables 9 and 10 show the results for Series I and II experiments respectively.

TABLE 9.

Expt. No.	8	15	9	11	10	14	7	4	12	5	18	17	13
*NaCl excreted	0·69	0·38	1·24	0·69	1·01	0·62	1·08	1·03	0·61	0·39	1·06	0·42	1·11
*NaCl injected	1·80	1·80	1·80	1·80	1·80	1·80	1·80	1·80	1·80	1·80	1·86	1·86	1·80
*NaCl retained	1·11	1·42	0·56	1·11	0·79	1·18	0·70	0·77	1·19	1·41	0·80	1·44	0·68
Normality of NaCl in urine	0·34	0·15	0·21	0·25	0·21	0·22	0·29	0·26	0·32	0·38	0·23	0·24	0·23
Body water loss	†3	6	62	11	51	12	26	31	†5	†20	43	†8	46

* Gm. per kg. † Indicates water retention.

TABLE 10.

Expt. No.	25	20	22	23	21	24
*NaCl excreted	0·51	0·64	0·69	0·91	0·69	0·70
*NaCl injected	1·86	1·86	1·86	1·86	1·86	1·86
*NaCl retained	1·35	1·22	1·17	0·95	1·17	1·16
Normality of NaCl in urine	0·24	0·20	0·20	0·18	0·18	0·19
Body water loss	6	18	21	49	29	25

* Gm. per kg.

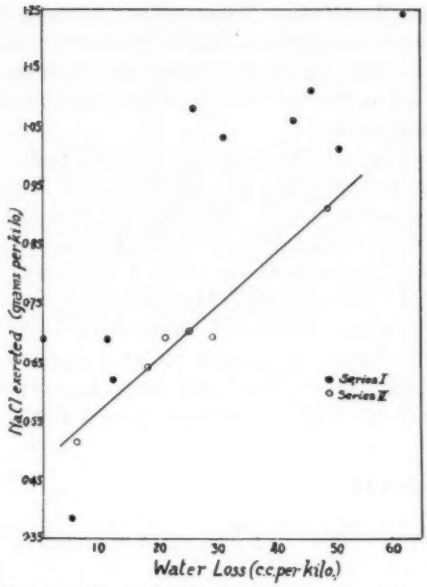

Fig. 3. Sodium chloride excreted and water loss.

Both series of results indicate a relationship between the NaCl excreted and the water loss per kilo, which when plotted shows, for Series II, a definite linear correlation (fig. 3). The points for Series I were also plotted (fig. 3), except where there was water retention, but it is not at all clear what type of relationship, if any, exists.

A correlation between urinary chloride concentration (normality) and water loss is also apparent. This relationship is shown in fig. 4, where the curves show the correlation between normality of NaCl in urine and water loss for Series I and II experiments respectively. This correlation is evidently an inverse linear one, i.e., the concentration of urinary chloride decreases with increasing water loss per kg.

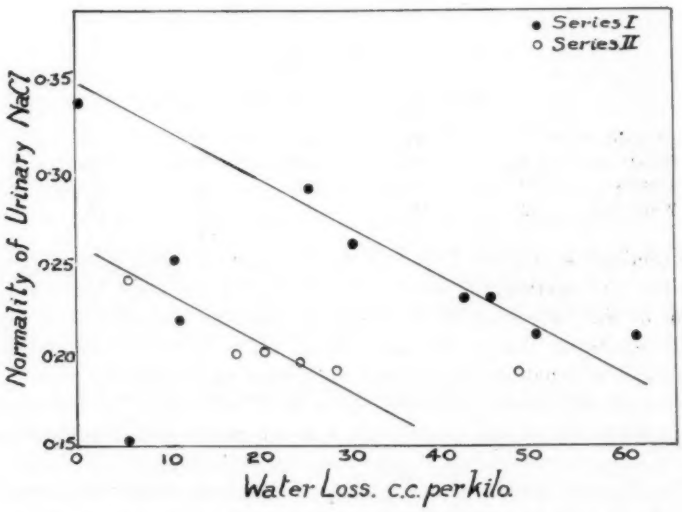

Fig. 4. Concentration of urinary sodium chloride and water loss.

Coupling the data from both figs. 3 and 4, it follows that, although the NaCl excreted does increase as water loss increases, the NaCl concentration does not keep pace with increasing water loss, i.e., there is a lag in the excretion of NaCl by the kidneys. This is supported by the fact that the blood chloride was abnormally high even at the conclusion of diuresis.

Some idea of the partition of the retained NaCl may be obtained, since the absolute increase in blood chloride can be calculated and the balance of the retained NaCl is presumably distributed in the tissues. The absolute increase in the NaCl of the blood of each rabbit was calculated from the NaCl increase per 100 c.c. blood (Tables 7 and 8) and the blood volume, the latter being computed from data given by Erlanger (1921), the mean of whose blood volume figures is 5·47 c.c. p.c. of the body weight. Subtracting the absolute increase in the NaCl of the blood from the total NaCl retained gives the amount of NaCl retained in the tissues. The partition of retained NaCl between blood and tissues is expressed as a percentage of the total salt retained, and is shown for both Series I and II in Tables 11 and 12 respectively.

TABLE 11.

Percentage Distribution of NaCl.

Expt. No.	5	17	12	8	15	11	14	7	4	18	13	10	9
Blood	5·1	7·6	8·7	7·5	4·6	10·4	8·5	13·0	—	9·6	14·0	14·3	19·4
Tissues	94·9	92·4	91·3	92·5	95·4	89·6	91·5	87·0	—	90·4	86·0	85·7	80·6
Water loss	*20	*8	*5	*3	6	11	12	26	31	43	46	51	62

* Indicates water retention.

TABLE 12.

Percentage Distribution of NaCl.

Expt. No.	25	20	22	24	21	23
Blood	8·5	8·4	6·7	7·5	8·3	9·3
Tissues	91·5	91·6	93·3	92·5	91·7	90·7
Water loss	6	18	21	25	29	49

Examination of Table 11 reveals the fact that there is a close relation between water loss and partition of NaCl, viz., the greater the water loss the smaller the amount of NaCl retained in the tissues at the time of cessation of diuresis. It may be significant that in the experiments where there was water retention the amount of NaCl that had passed into the tissues was uniformly high.

No such correlation exists for Series II (Table 12). The partition of NaCl between blood and tissues is almost a constant proportion, irrespective of water loss.

This passage of excess NaCl into the tissues, presumably in a direction opposite to that in which water is leaving, is related to a phenomenon noted by Marie

(1930) and also by Ambard and Beaujard (1905), namely, historetention or the dry retention of NaCl, in which the excess NaCl is believed to be bound to the tissues in an osmotically inactive form.

URINARY pH.

The urinary pH was determined in only a few experiments of both series, and the results are given in Table 13, together with the plasma CO_2 capacities. Two pH measurements were made during the experiments, the first on urine passed in the first 45 minutes of the experiment, and the second on urine passed in the following 45 minutes. pH values are separated by an arrow, indicating the serial order of the measurements.

TABLE 13.

	Series I.				Series II.		
Expt. No.	15	14	18	17	22	23	24
Plasma CO_2 before injection	43·7	47·4	55·7	66·9	63·3	61·2	73·8
Plasma CO_2 after diuresis	37·8	45·9	53·6	49·0	52·8	61·2	70·7
Mean Plasma CO_2	40·8	46·7	54·7	58·0	57·8	61·2	72·2
Urinary pH	5·25→5·07	8·23→7·74	7·30→7·40	8·34	6·30→7·10	6·68→7·02	6·98→7·45

The pH of the first 45-minute sample of urine reflects the mean alkali reserve of the blood. In Series I the urinary pH fell more to the acid side, as did also the alkali reserve, whilst in Series II the urine always became more alkaline, in spite of a fall in the alkali reserve of the blood. In experiments 23 and 24, where lactic acid was injected, no acidosis resulted and no excretion of lactic acid followed. This is supported by the experiments of Abramson and Eggleton (1927), who observed utilization of intravenous sodium lactate by dogs, and who record that saline diuresis produces a lowering in blood bases and an excretion of bases in the urine; while Hendrix and Calvin (1925), in similar experiments with NaCl in dogs, observed an increased loss of base through the kidney and a notable fall in alkali reserve. Hendrix and Calvin, however, attribute the loss of base to inefficient reabsorption by the overtaxed tubules.

OSMOTIC PRESSURE OF SERUM COLLOIDS.

The following values (Table 14) for the osmotic pressure of the serum colloids are given for comparative purposes only. Satisfactory observations for Series II only were obtained, and the osmotic pressure is expressed as millimetres pressure of water.

TABLE 14.

Expt. No.	20	22	23	21	24
Os. Pr. before injection	230	170	335	284	289
Os. Pr. after diuresis	190	217	301	280	289

The experiments are placed in order of ascending plasma CO_2 capacity. With increasing alkali reserve the osmotic pressure rises up to a maximum value (experiment 23) and then falls again with still increasing alkali reserve. The number of observations made is inadequate, but accords with the observations of Loeb (1922 and 1923).

It has been shown by S. P. Sörensen (1915-1917) that egg-albumin solutions of constant composition invariably exerted the same osmotic pressure, and that diminution of osmotic pressure was effected by the presence of salts (ammonium sulphate in his experiments), possibly due to condensation of the protein molecules by the latter. These facts lend some significance to the figures in Table 14. The fall in osmotic pressure may be due to condensation of the serum proteins by the hypertonic NaCl. This hypothesis is supported by the fact that the blood chloride was abnormal at the cessation of diuresis, and our analyses would not reveal whether the extra NaCl was bound to protein or was in free solution. The fall in osmotic pressure was irregular and does not correlate with any other factor.

CONCLUSIONS AND DISCUSSION.

The experimental evidence points to a direct proportionality between the alkali reserve and the water loss per kilo., i.e., the reserve water of the body. This, therefore, supports the conclusions of Andrews (1926). The curves (see fig. 1) expressing the above correlation for the two series of experiments lie very approximately parallel to one another, indicating that the dietary difference *per se* had no effect upon the amount of reserve water. The diverging of two points, evidently caused by the simultaneous injection of lactic acid, shows how easily the water balance may be disturbed, for presumably the lactic acid was immediately metabolized, no acidosis being manifested. The work of Abramson and Eggleton has been cited in support of this conclusion, while the urinary pH and tissue dehydration values in these divergent experiments accord with the hypothesis.

The divergencies in Series I may be due to metabolic interference. Mathison (1910), whilst examining asphyxia in spinal cats, elicited spontaneous irruption of reflexes by the much more rapid injection of 5 c.c. of N/10 lactic acid in a period of 15 secs.

The evidence points to a slight dilution of the blood at the time of cessation of diuresis, a dilution which probably was much greater during the course of saline infusion. A slight shrinkage of the corpuscles was indicated, which is to be expected as a direct effect of both the hypertonic saline and operation of the

Donnan law. Krevisky (1930) has shown that 30–35 p.c. of the cell volume of erythrocytes is occupied by free or osmotically transferable water; if in our experiments the corpuscular shrinkage be taken as a measure of water which has been osmotically transferred, the former bears no relation to the body water loss. It is assumed, therefore, that this free water is not an important factor in our problem. The part that the dilution due to withdrawal of blood plays in the subsequent water exchange can only be speculative in the present state of knowledge of such equilibria. Concerning the water content of muscle tissues, no definite conclusions can be drawn from the first series of experiments, but in Series II there was a dehydration of muscle correlated with the body water loss; and computation showed that the major part of the body water loss could be accounted for by the extent of dehydration of the muscle, which tissue, it is concluded, holds most if not all of the "reserve water". This conclusion is in agreement with the findings of Skelton (1927). On the other hand H. Schade (1925) attributes important water-depot functions to the connective tissues. There is no doubt that the water removed in Series I, where diet was uncontrolled, was ultimately derived from the intestine, although the influence of alkali reserve upon water loss indicates that the mode of removal was not simple.

Effects due to the anaesthetic cannot be excluded, but Molitor and Pick (1925) have shown that paraldehyde itself does not affect a water diuresis.

Although the increase in blood chloride was very great, it is doubtful whether this increase amounts to hypertonicity of the blood, because there is a constant tendency towards ionic equilibrium (Donnan equilibrium), and hence the excess Cl ions would cause the organism to rid itself of ions such as HCO_3^-. Essen, Kauder, and Porges (1923) showed that changes in plasma chloride were accompanied by reciprocal changes of the bicarbonate, estimated indirectly by measurement of the alveolar CO_2. This may be due to the fact, shown by Iversen (1924) that when absorbed chloride diffuses into the tissues it is accompanied by the same relative amount of bicarbonate as exists contemporaneously in the blood, thus lowering the bicarbonate content of the latter. At the same time the kidneys remove bicarbonate. Both these results may contribute to the fall in the CO_2 combining capacity of the blood found during the experiments. The trend of urinary pH to the alkaline side in Series II experiments supports the above findings.

A difference between the water metabolism of the two series is indicated by the relationship between the NaCl excreted and the water loss per kg., which was linear for Series II and possibly curvilinear for Series I (fig. 3). Moreover, in the partition of the retained NaCl, the percentage of NaCl in tissues other than blood was inversely proportional to the water loss for Series I, whilst for Series II no such relationship existed, the partition in this case being an almost constant proportion.

It is noteworthy that the correlation between concentration of urinary NaCl

and water loss, in both series of experiments, is an inverse linear one and that the two curves (fig. 4) are parallel. The kidney function in both series was therefore a constant factor, again indicating that the anomalies of Series I were extrarenal in character.

As already stated, there was a lag in the excretion of NaCl as compared with the water excretion by the kidneys, and this points to an independent excretion of NaCl and water. Furthermore, it is evident that whilst water was passing out from the body tissues, NaCl was also passing inwards; but in the light of Skelton's researches the hypertonic saline probably permeated all the tissues excepting muscle. During this passage NaCl would be retained (historetention) by the skin and connective tissues, whilst water was withdrawn from the muscle tissue by osmotic forces, the saline thereby becoming diluted. The extent of water reserve in the muscle, the extent of dilution of the saline and hence of the blood, and the consequent diuresis depend upon the alkali reserve of the blood. The few successful measurements of the osmotic pressure of the serum colloids possess no obvious significance, but Howe (1925), in a review of the studies of swelling pressure of plasma proteins made by Schade, states that the hydration of the proteins is involved in the water balance.

SUMMARY.

1. A standardized method for the production and observation of experimental diuresis in rabbits has been established.

2. Estimations of blood alkali reserve, blood dilution, blood chloride, tissue water, urinary chloride, urinary pH, and osmotic pressure of serum colloids were performed on samples taken both before the beginning and at the end of experiments.

3. The water loss (calculated from the observed diuresis) was found to be related to the alkali reserve of the animal's blood, viz., the water loss was increased by 2·9 c.c. per kg. for each c.c. increase in the CO_2 combining power of the plasma. This water loss is a measure of the reserve water of the body.

4. This reserve water is stored chiefly in the muscle tissues.

5. The effect of dietary control upon this correlation was investigated, and it appears that the metabolism (e.g., of lactic acid) affects the availability of the reserve water.

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THE EFFECT OF PITUITRIN ON THE WATER BALANCE OF RABBITS

by

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The following investigation was undertaken for the purpose of discovering whether whole extract of the posterior lobe of the pituitary had any effect upon the physiological factors determining the distribution of water between blood and tissues, when the latter were subjected to the dehydrating influence of an intravenous infusion of hypertonic saline.

Previous work of Cox and Hicks (1933b) had demonstrated the existence of a linear relationship between the alkali reserve of the plasma and the free water in the tissues, i.e., water which is removable by intravenous injection of hypertonic saline. The same experimental procedure was employed, with the exception of the addition of pituitary extract to the infusion of fluid, and some analytical modifications as reported by Cox and Hicks (1933a) in another paper.

Rabbits, fed for the previous four or five days on the prescribed dry diet, were infused with 5 p.c. saline at the rate of 25 c.c. per hour for 90 minutes, and pituitrin was added to the saline so that 250 c.c. saline contained 1 c.c. of Parke, Davis & Co.'s pituitary extract. The animals therefore received 0·1 c.c. of pituitrin per kilo. per hour.

TABLE 1.

Expt. No.	31	32	35	33	45	38	36	34	40	37	42
CO_2 combining power† before injection	*25·1	34·4	44·1	46·2	51·1	47·0	45·8	48·9	58·2	53·1	54·3
CO_2 combining power† after diuresis	20·1	*27·5	27·2	33·0	31·2	39·5	42·8	42·2	35·6	42·0	48·7
Mean CO_2 combining power†	*22·6	*31·0	35·6	39·6	41·1	43·2	44·3	45·5	46·9	47·6	51·5
Water retention	32	24	—24	16	4	19	—7	13	—12	4	—57

* These figures are only approximately correct. † c.c. of CO_2 per 100 c.c. plasma.

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In seven out of eleven experiments water was retained by the tissues of the animals; moreover the amount of water retained per kilo. bore a definitely linear relationship to the mean alkali reserve (see fig. 1). The alkali reserve and water retention figures are shown in Table 1.

In four experiments, 35, 36, 40, and 42, there was a water loss of unexpected magnitude (indicated by the negative sign), and they were therefore, in this respect, anomalous. However, the other experiments show beyond doubt that there has been an inhibition of water loss, i.e., of diuresis, because instead of the water loss usually induced when the alkali reserve of the plasma exceeds the critical value of 45 c.c. p.c., there was actually water retention with an alkali reserve of the order of 50 p.c. It would seem that the effect of the pituitrin has been to cause an apparent raising of the critical level of alkali reserve and an inhibition of water loss.

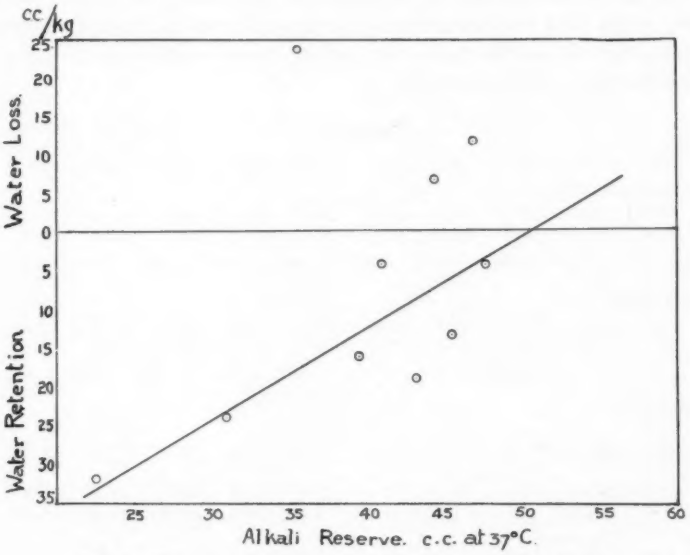

Fig. 1. Reserve water and alkali reserve after pituitrin.

If the curve of fig. 1 be compared with the similar curve of Series II experiments in our previous communication (Cox & Hicks, 1933b), it will be noticed that the slope of the former curve is less abrupt than that of the latter curve. This evidently indicates that with increasing alkali reserve, and consequently with a greater tendency to water loss, the inhibitory effect of pituitrin would become more marked, both absolutely and relatively. Since, in the present series, the decrease in water retention is almost 1·2 c.c. per kilo. for each c.c. of increase

in the CO_2 combining capacity of the plasma, and the corresponding value for our previous curve was 2·9 c.c., we infer that pituitrin has caused an inhibition of diuresis to the extent of about 1·7 c.c. per kilo. for every c.c. increase in the CO_2 combining capacity of the plasma.

Another effect observed in these experiments was the large fall in alkali reserve (a mean fall of 20 p.c.) during the course of the experiment. In this connection Nitzescu and Munteanu (1931) have reported an increased production of lactic acid in the muscles of both dogs and rabbits, due to intravenous injection of pituitrin. This production of lactic acid may account for the large fall in alkali reserve noticed in these experiments.

When we examine the figures for blood dilution, viz., the blood iron contents and the corpuscular volumes, and also the analyses of the water content of muscle tissue (Table 3), we find further evidence in support of the above conclusion For the sake of comparison the retention of water by the body (as c.c. per kilo.) is included, along with the theoretical water retention or loss computed from the muscle water figures, on the basis that muscle tissue makes up 52·2 p.c. of a rabbit's body weight (Skelton, 1927).

TABLE 2.

Expt. No.	31	32	35	33	45	38	36	34	40	37	42
Blood Iron (mg. per 100 c.c.)											
Fe_1	61·5	55·0	47·2	71·4	47·6	50·3	56·4	54·0	44·6	53·0	37·8
Fe_2	49·6	38·9	40·9	59·8	43·2	49·8	56·4	47·3	43·5	50·6	39·6
$\frac{Fe_2}{Fe_1}$	0·807	0·707	0·867	0·838	0·908	0·990	1·000	0·876	0·976	0·955	1·05
Corpuscular Volume.											
V_1	—	—	32·8	42·0	38·3	46·4	50·0	47·6	35·1	45·4	34·2
V_2	—	—	30·3	36·1	28·2	38·1	45·0	35·2	30·8	40·1	34·9
$\frac{V_2}{V_1}$	—	—	0·924	0·860	0·736	0·821	0·900	0·740	0·878	0·883	1·02
Tissue Water.											
Before injection	73·2	72·5	73·6	72·3	76·7	73·9	72·4	73·1	73·4	73·6	75·1
After diuresis	71·4	67·3	73·0	73·3	74·4	71·5	71·5	73·2	71·9	75·8	72·7
Loss	1·8	5·2	0·6	+1·0	2·3	2·4	0·9	+0·1	1·4	+2·2	2·4
Body water retention	+32	+24	—24	+16	+4	+19	—7	+13	—12	+4	—57
Computed water loss	—9	—27	—3	+5	—12	—13	—5	+1	—7·8	+12	—12·5
Liver Water.											
Before injection			73·1			73·1	73·1				
After diuresis			58·7			65·2	72·0				

Fe_1 = Iron content of the blood before saline infusion.
Fe_2 = Iron content of the blood after cessation of diuresis.
V_1 = Corpuscular volume prior to infusion.
V_2 = Corpuscular volume after cessation of diuresis.
+ sign indicates water retention.

The extent of blood dilution as measured by the ratios $\frac{Fe_2}{Fe_1}$ and $\frac{V_2}{V_1}$ follows approximately the extent of water retention or even of water loss, i.e., the greater the water retention the greater the blood dilution. This is made more evident when the ratios of the iron values and of the corpuscular volumes are plotted against water retention per kilo. (see fig. 2). Inspection of the figures (Table 2) or of fig. 2 reveals the fact that the ratio of the corpuscular volumes is smaller than the corresponding iron ratio which must be taken to indicate shrinkage of the corpuscles, and that the shrinkage is greater the greater the water loss. Moreover, if these blood dilution figures be compared with those in our previous paper, they will on the whole be seen to signify a greater dilution than when no pituitrin was present. From this we infer that the excess of water over that normally retained or lost for any given CO_2 combining capacity has been held back in the blood-stream; that is, that the inhibition of diuresis is renal in character.

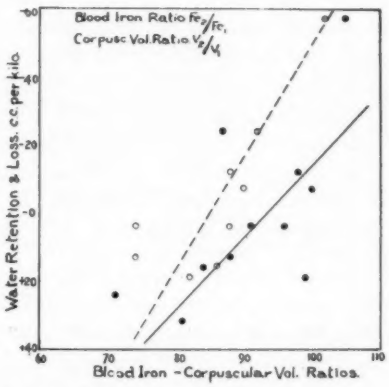

Fig. 2. Blood dilution and water retention.

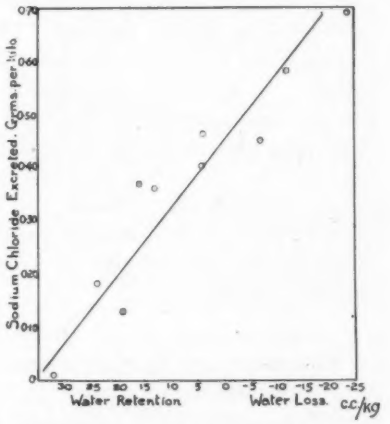

Fig. 3. Sodium chloride excreted and water loss.

When we examine the water-balance of the muscle tissue we find conflicting data; for instance, in experiments 33, 34, and 37 the tissue actually increased in water content, whereas in the remaining experiments there was a variable water loss. The theoretical values for water retained or lost from the body, computed from the tissue analyses, however, seem to bear no relation to the actual retention of water by the body. It is rather remarkable to find such great loss of water from the muscle tissues in experiments 31, 32, 38, and 45, in spite of extensive water retention by the body; these losses are quite out of keeping with the mean

alkali reserve, and we are tempted to conclude that they indicate an extra-renal effect of pituitrin.

Only a few analyses of liver water were performed, but it is evident that they tend to follow the same course as the muscle tissue figures (Table 2).

Examination of the chloride analyses for the blood, muscle tissues, and urine (Table 4) reveals some marked differences from the chloride balance observed in our previous work. Thus, the increase in blood chloride in the pituitrin experiments is considerably greater than in the ordinary saline infusions (cf. Tables 7 and 8, Cox & Hicks, 1933b). On plotting NaCl excreted per kilo. against water retained per kilo. (fig. 3) it is found that an extra 0·13 gm. NaCl is excreted per kilo. for each 10 c.c. decrease in water retention; but, comparing these facts with the corresponding data in fig. 3 of our previous paper we find that 0·10 gm. NaCl was excreted per kilo. for each 10 c.c. increase in water loss. From this it is inferred that pituitrin has caused an added excretion of NaCl beyond the amount normally excreted. The figures for the normality of the urinary NaCl show that in nearly every case (save in the anomalous experiment 35) the concentration of NaCl increases progressively from Sample I to Sample III (samples collected in 45-minute periods). This indicates independent excretion of water and chlorides by the kidneys, an observation already made in our first communication. The figures for chloride in the muscles show that, during the saline infusion, a small amount of NaCl has entered the muscle cells, a fact not entirely in keeping with Skelton's conclusion (1927) that muscle is non-permeable to the chlorine ion. However, pituitrin may be an unknown factor in inducing this muscle permeability, although the increase in chloride content of the muscles tends to decrease in a manner parallel with decreased retention of water. The latter figures are included in Table 3 for purposes of comparison with the chloride balance.

TABLE 3.

Expt. No.	31	32	35	33	45	38	36	34	40	37	42
NaCl excreted (gm. per kg.)	·007	0·18	1·25	0·50	0·46	0·25	0·88	0·45	0·58	0·60	1·30
NaCl retained (gm. per kg.)	1·90	1·73	0·66	1·41	1·51	1·66	1·03	1·46	1·39	1·31	0·67
Normality of urinary											
NaCl I		0·27	0·21	0·25	0·22	0·09	0·19	0·21	0·20	0·27	0·22
NaCl II	0·02	0·21	0·19	0·33	0·27	0·22	0·20	0·25	0·21	0·21	0·22
NaCl III		0·26	0·17	0·32	0·21	0·25	0·26	0·31	0·22	0·26	0·28
Blood Chloride (mg. per 100 c.c.)											
Before injection	—	—	463	363	438	450	438	350	425	406	438
After diuresis	—	—	700	650	763	700	575	600	713	625	600
Increase	—	—	237	287	325	250	137	250	288	219	162
Muscle Chloride (mg. per 100 c.c.)											
Before injection	26	50	126	102	150	69	116	91	73	96	86
After diuresis	90	148	104	133	129	118	136	119	135	115	136
Increase	64	98	—22	31	—21	49	20	28	62	19	50
Body water retention	+32	+24	—24	+16	+4	+19	—7	+13	—12	+4	—57

Several osmotic pressure readings of the serum colloids were taken on blood drawn at the end of diuresis, and the values for the several experiments were all about 135 mm. of water. No blood was taken for osmotic determinations before the injection period, but if the above figure be tentatively compared with the mean value for osmotic pressure (before injection) of our earlier work, namely, about 280 mm., a very large drop in the osmotic pressure of the serum colloids is evident. At the present stage of our work we are not prepared to estimate the significance of the above data, but they certainly agree with the similar observations reported by Tada and Nakazawa (1930).

DISCUSSION.

A survey of the recent literature on the action of pituitrin upon water metabolism shows that most of the researches on this question reveal the anti-diuretic action to be predominant. Thus the work of Roboz (1931) and McIntyre and Van Dyke (1931) shows definitely an inhibition of diuresis, accompanied by marked hydraemia, as in the present instance. Roboz also believes that the hyperaemia is due not only to water resulting from the inhibition of diuresis, but also to an extra-renal separation of water from the tissues, of which we have also obtained some evidence. Roboz, and McIntyre and Van Dyke favour the view that the anti-diuretic effect of pituitrin is renal in character; the latter workers also remark upon the increased concentration of chlorides in the blood and urine, as we observed to be the case in our experiments. Underhill and Pack (1923), Craig (1925), Hines, Leese and Jacobs (1927) all observed the anti-diuretic effect of pituitrin upon fluid injected intravenously. Craig, however, reports diuresis in anaesthetized dogs, when subcutaneous injection of pituitrin is followed by peroral administration of water, although Buschke (1928) obtains inhibition of diuresis in peroral experiments. Robert (1932), too, reports anti-diuretic effects of pituitrin when water is perorally administered to human subjects. He also examines the effect of the pressor and oxytocic principles upon the water-salt metabolism, and finds that the former has the same effect as pituitrin itself, but that the latter is much weaker in its action. Robert finds no increase of NaCl in his experiments, although dilution of the blood is very marked.

Our observation that the excretion of water and of salt by the kidney occur independently of each other, is borne out by similar observations made by Buschke (1928) and by Hirsch (1931) in their experiments on diuresis.

SUMMARY.

1. Rabbits were infused with 5 p.c. saline containing 0·4 p.c. pituitrin at the rate of 25 c.c. per kilo. per hour. Inhibition of the diuresis was observed to the extent of about 1·7 c.c. per kilo. for every c.c. increase in the CO_2 combining power of the plasma.

2. Blood dilution proportional to the water retention was a concomitant of the inhibition of diuresis, leading to the conclusion that the effect of pituitrin is renal in character.

3. Muscle tissue analyses presented some evidence of an extra-renal separation of water by pituitrin.

4. Increased excretion of chlorides occurred as a result of injections of pituitrin.

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